Olink® Focus

User Manual

1231, v1.4, 2023-11-17

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1. Introduction

1.1 Intended use

The custom-made Olink® Focus multiplex immunoassay panels provide an efficient and innovative tool for targeted human protein biomarker validation and implementation. Use our standard offer of Olink® Explore and Target panels to find a set of biomarkers defining your protein profile of interest. Once you have established a set of biomarkers of interest, we can work with you to design and validate a smaller panel of 15–21 assays. Moreover, you can choose between relative quantification or absolute quantification, as well as select the level of panel performance characterization based on your requirements.

1.2 About this manual

The Olink Focus User Manual provides you with the instructions needed to run an Olink Focus 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 two 96-well plates prior to the start of the run.

1.3.2 Sample types

Olink Focus panels have been validated as agreed with the customer for the specific panel, usually with EDTA plasma and serum samples. Different sample matrices are expected to affect the detection of specific proteins in different ways. Extreme levels of IgG or fluorescent particles can interfere with the Olink assay. For more information on sample types, please see the panel-specific validation data document provided to the customer, 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 all pipettes regularly.

1.4 Associated documentation

1.4.1 Olink documentation

The Olink documents listed below 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"

Material Safety Data Sheet

- Olink® Focus Detection kit
- Olink® Focus Control kit
- Olink® Focus Calibrator kit
- Olink® Focus Probe kit

1.4.2 Other documents

For information on the Standard BioTools™ BioMark System, read the following User Guides that can be found at www.standardbio.com/:

- Fluidigm™ Real-Time PCR Analysis User Guide — PN 68000088
- Fluidigm™ Data Collection Software User Guide — PN 68000127

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

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

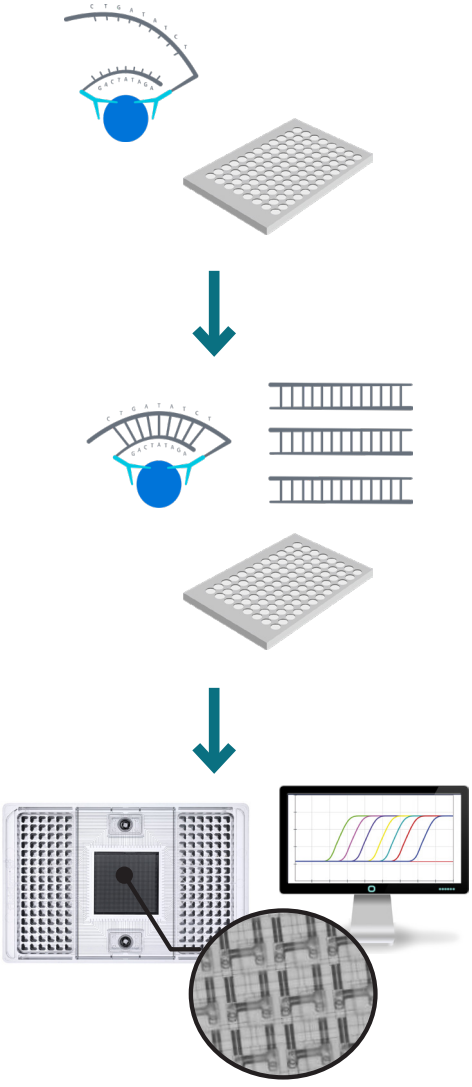
1.5 Technical support

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 antibodies labelled with DNA oligonucleotides, bind to their respective protein in the samples.

2. Extension and amplification

Duration: 2 hours

Matching 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: 2.5 hours

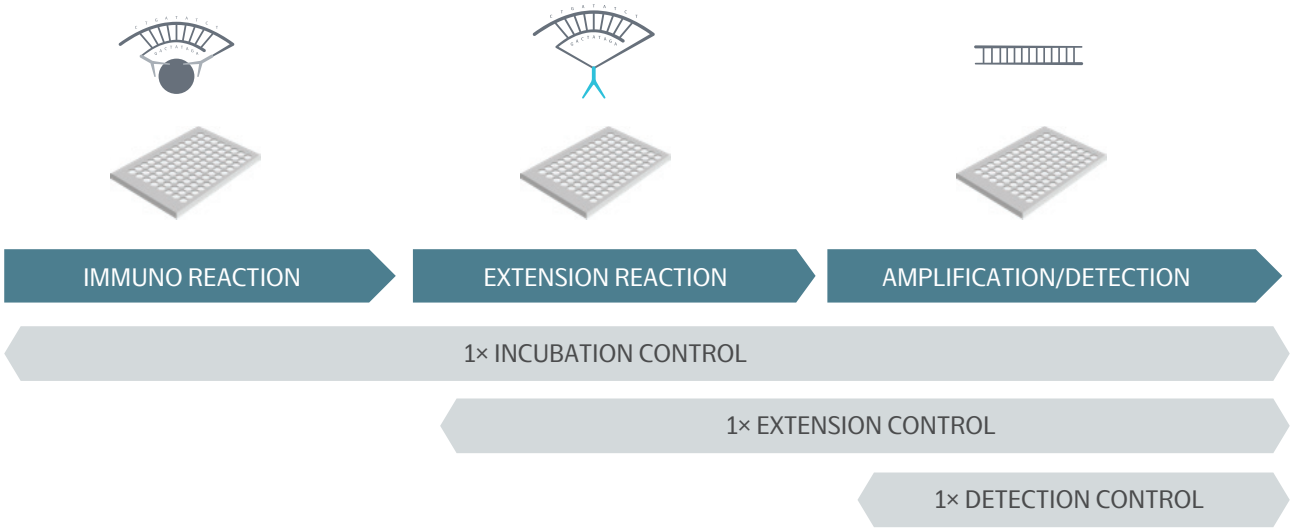
The amount of each unique DNA barcode is quantified by microfluidic qPCR.

2.2 Quality control

Olink has developed a built-in Quality Control (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 three 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.



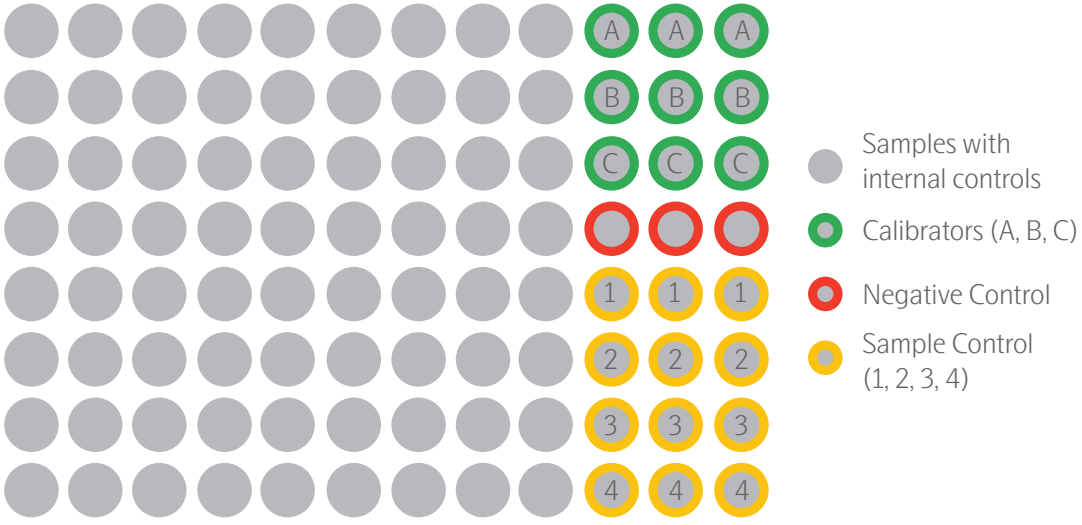
Incubation control: The Incubation Control is a non-human antigen measured with PEA. This control monitors potential technical variation in all three steps of the reaction.

Extension control: The Extension Control is composed of an antibody coupled to a unique pair of matching 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 in the normalization process of each sample.

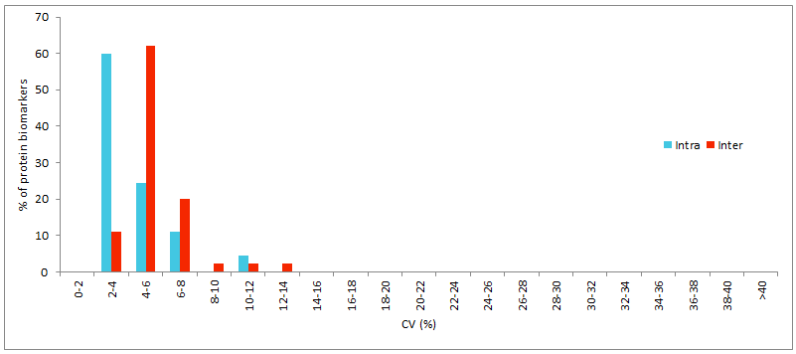
Detection control: The Detection Control is a complete double stranded DNA amplicon which does not require any proximity binding or extension step to generate a signal. This control monitors the amplification/detection step.

2.2.2 External controls

There are three required external controls that are added in triplicate or duplicates on each sample plate. On the following illustrated plate there are 72 samples and four Sample Controls, one Negative Control and three Calibrators in triplicate.



Sample Control: The Sample Controls are usually customer samples of the same sample type that the panel was validated for. They are there to ensure that all proteins are detected as expected. The Sample Controls are used to assess potential variation between runs and plates, as well as quality control of the quantification of data in pg/mL. See the Results sections below. An example of inter- and intra-assay CV is illustrated here:



Negative Control: Negative Control is included in triplicate on each plate and consists of buffer. 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) for each plate, which is defined as three fixed standard deviations above average for the negative controls.

Calibrators: The Calibrators consist of recombinant antigens for all the proteins the panel was developed for. The calibrators are included in triplicate on each sample plate and used to normalize all samples to NPX (see *2.2.4 Results in relative unit (NPX)*) or pg/mL based on a pre-defined standard curve (see *2.2.5 Results in standard unit (pg/mL)*).

2.2.3 Data analysis

Protein expression is reported in standard concentration units (pg/mL) or relative Normalized Protein eXpression (NPX) units depending on your choice. Quality control and normalization is achieved using the Olink NPX Signature software, refer to *4.1 Olink® NPX Signature*.

2.2.4 Results in relative unit (NPX)

Olink reports protein expression levels using an arbitrary unit called Normalized Protein eXpression. 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 for relative quantification Olink Focus panels:

Each sample is normalized with its corresponding extension control level:

$$Ct_{\text{Sample i, Assay j}} - Ct_{\text{Sample i, Assay Ext ctrl}} = dCt_{\text{Sample i,Assay j}}$$

Each plate is normalized with its Calibrators to generate NPX levels and adjusted against a pre-determined bridging factor.

$$\text{Bridging factor}_{\text{Assay j}} - (dCt_{\text{Sample i, Assay j}} - \text{Mean}(\text{Median } dCt_{\text{Calibrator A, Assay j}}; \text{Median } dCt_{\text{Calibrator B, Assay j}}; \text{Median } dCt_{\text{Calibrator C, Assay j}})) = NPX_{\text{Sample i,Assay j}}$$

NOTE: *The NPX value will differ between the same protein measured on Olink Target 96, Olink Target 48 or Olink Explore mainly due to different use of bridging factor and correction factor, and different samples used for plate normalization.*

The NPX unit is unique to each protein assay, meaning that even if two different proteins have the same NPX values, their concentrations in pg/mL may differ.

2.2.5 Results in standard unit (pg/mL)

The method for quantification of data in standard units (pg/mL) for absolute quantification Olink Focus panels is described in this section.

Method overview

Before run (during product development at Olink)

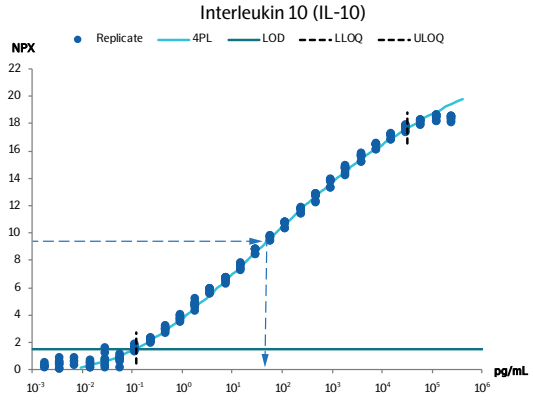
1. A precise pre-defined standard curve is established for each protein.
2. The calibrators are used to adjust the standard curve along the y-axis to normalize between plates and a second normalization, utilizing a bridging factor, is applied to ensure accuracy between production batches.
3. A 4-PL model fit is performed to define the standard curve mathematically in the measurement range for each protein in the panel.

At each run

1. The median value of each calibrator triplicate is used to adjust the measured samples along the y-axis to normalize between plates. The samples are also adjusted against the bridging factor.
2. The measured sample values are related back to the adjusted standard curve model which is used to translate the measured NPX value to the protein concentration in pg/mL.

Standard curve

During development, a thorough fine-tuned 32-point standard curve is developed for each protein biomarker simultaneously including all proteins included in the panel. Multichannel pipetting and numerous replicates of the curves are used to minimize errors and establish an accurate immunoassay curve fitting. Due to the wide dynamic range of the PEA assays in the panel, a large spread of measurement points are required to cover the entire range. Eight measurement points, which is commonly used in many technologies, would not suffice for Olink's multiplex measurement of up to 21 assays. The predefined standard curves avoid operator dependent reconstitution and pipetting of standard curves at each lab and for each run. The figure below shows an example of a point standard curve defined for each assay during development. Standard curves for each assay can be shared with the customer on request.



4PL model

A 4PL model curve fit is used to describe the immunoassay standard curve, indicated by the turquoise line in the figure above. When running a project, the measured patient sample value (represented by the dotted blue arrows in the figure) is related back to the adjusted standard curve model which translates the measured value to the protein concentration in pg/mL. Repeated testing and validation show that the 4PL curve fitting describes the standard curve well, and can be used to correctly estimate the concentration in analyzed samples within the LOQ. The lower and upper limits of quantification (LLOQ and ULOQ) are defined during the development of the panel, and shared with the customer after panel finalization.

QC of assays in pg/mL

The QC of each assay is performed utilizing the Sample Controls. They are assessed for both accuracy and precision (CV%) for each individual assay to confirm that the concentration values in pg/mL are within the expected limits (±30% of expected value) and assure the correct functionality of the quantification method.

3. Product description

3.1 Reagents supplied in Olink® kits

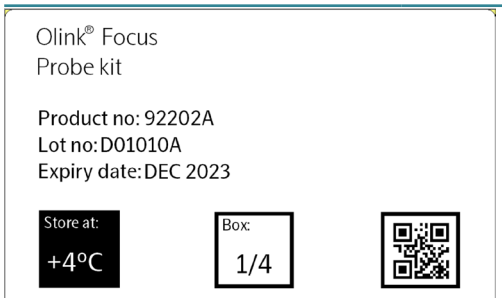
Each Olink® Focus kit contains reagents for two 96-well plates, meaning two times 72–80 samples and 16–24 controls. Both plates will be combined on the same Olink® 24.192 IFC (integrated fluid circuit) in the final detection step. The reagents are supplied in three individual boxes. Storage temperature and expiry date for the components are stated on the label on each box.

NOTE: *All reagents for Olink® Focus kits are lot specific and reagents from different kit lots cannot be combined. Do not use reagents from Olink® Target or Explore kits with reagents from the Olink Focus kits.*

3.1.1 Probe kit

The Olink Probe kit should be stored at +4 °C. The Probe kit includes:

Part	Description
Incubation Solution	Contains components needed for the immuno reaction
Frw-probes	Contains up to 21 antibody probes labeled with Frw-oligos
Rev-probes	Contains up to 21 antibody probes labeled with Rev-oligos

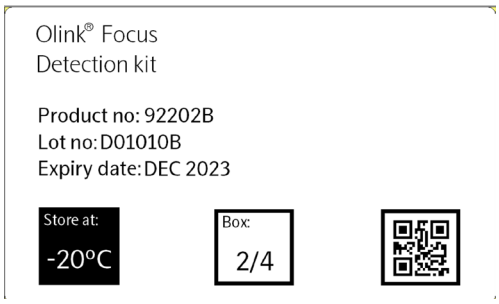


3.1.2 Detection kit

The large box contains the Olink Detection 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 Frw- and Rev-probes bound to their target
PCR Polymerase	For pre-amplification of the extension product created by the PEA Enzyme
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
Negative Control	For determination of background levels

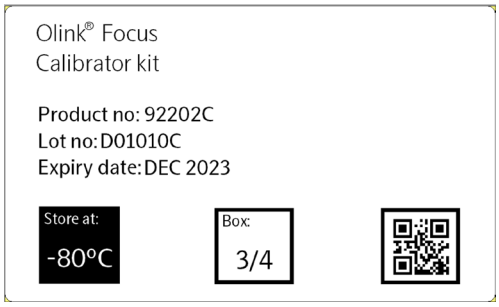
PEA Enhancer	Contains components needed for the extension reaction



3.1.3 Calibrator kit

The Olink Calibrator kit should be stored at -80 °C. The Calibrator kit includes:

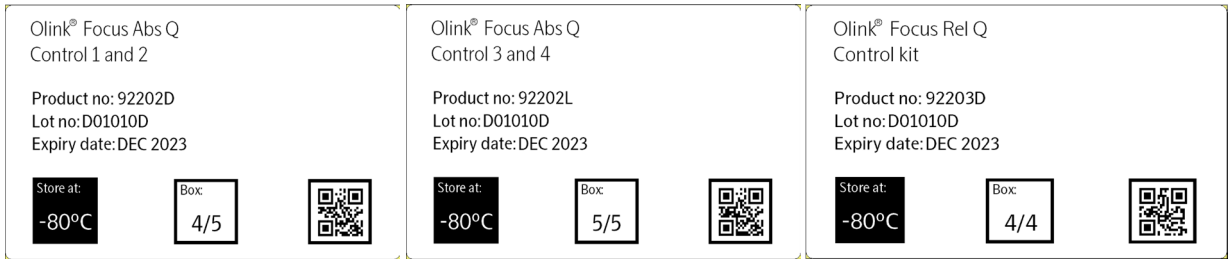
Part	Description
Calibrators	Used for normalization between plates and of samples



Control kit

The Olink Control kit should be stored at -80 °C. The Control kit includes:

Part	Description
Sample controls	Used for quality control of quantification of data in pg/mL and to assess variation between runs and plates. For Focus AbsQ the controls are delivered in two separate boxes.



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 Guide for more information.

4.2 Required consumables (not supplied)

- Pipette filter tips
- Microcentrifuge tubes (1–1.5 mL)
- Centrifuge tube (50 mL)
- 8-well strips with lids
- Four (six for diluted panels) 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® 24.192 IFC for Protein Expression

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 Eppendorf® 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)

NOTE: *The workflow can be performed with one or two thermal cyclers. See further information in section 8.1 Overview.*

- 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

- Freezer (-80 °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

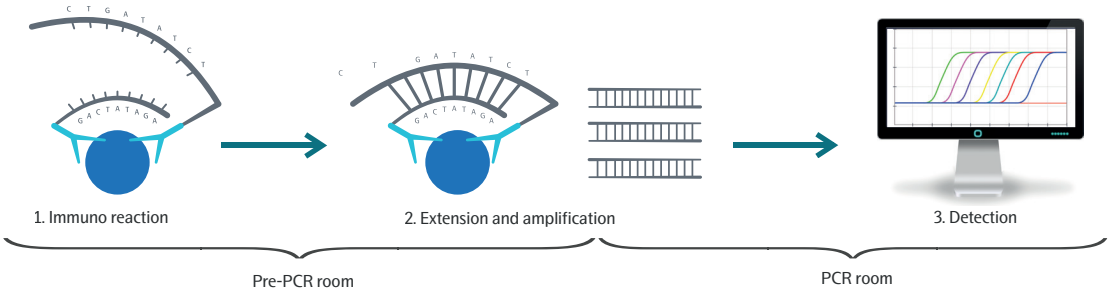
Follow general laboratory safety procedures:

- Use gloves, safety goggles and protective clothing when performing the experiments.
- Handle solutions with particular caution, as Dimethyl sulfoxide (DMSO) is known to facilitate the entry of organic molecules into tissues. DMSO is included in PEA Enhancer.
- Handle and dispose of hazardous sample material according to local regulations.

For complete safety information, refer to Material Safety Data Sheets (MSDS) available on the Olink website: www.olink.com/downloads.

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 Olink Signature Q100 instruments regularly.



5.2 Pipetting guidelines

5.2.1 Tips and tricks for pipetting

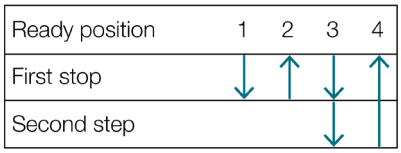
- Calibrate pipettes regularly (at least a 6 month interval)
- Pipette near the liquid surface
- Let the reagents and liquids reach room temperature before use to maximize accuracy
- Pre-rinsing the tip 1 to 3 times with the liquid to be pipetted improves accuracy when using reverse pipetting
- Do not 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, unless otherwise stated in the protocol

5.2.2 Pipetting techniques

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

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

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. Ensure that all tips contain the exact same volume if a multichannel pipette is used.
3. Dispense the liquid into the receiving vessel by gently pressing the operating button to the first stop 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 to the ready position.

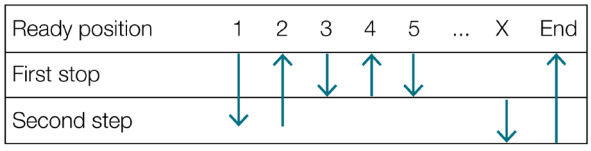


Reverse pipetting improves precision with smaller volumes and viscous solutions and is performed as follows:

5. Press the operating button past the first stop.
6. Dip the tip into the solution to a depth in accordance with the volume set, and slowly release the operating button. This action will fill the tip with a volume that is larger than the set volume.

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

7. Remove the tip from the liquid and dispense the liquid into the receiving vessel by pressing the operating button gently and steadily to the first stop. This volume is equal to the set volume.
8. Hold the button in this position. Some liquid will remain in the tip- this should not be dispensed.
9. Continue pipetting by repeating steps 3 and 4.



5.3 Vortexing guidelines

Correct vortexing is critical when running Olink panels.

5.3.1 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.

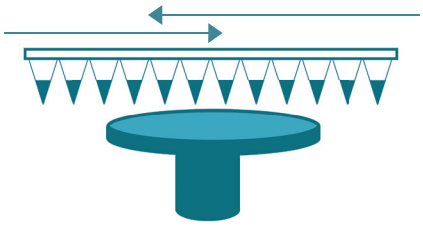
- Set mixing speed at 2,000 rpm and mixing time at 30 seconds.
- Start mixing by pressing the start/stop key. After 30 seconds, MixMate® will automatically stop.
- Take out the plate by pulling it out from the front side of the plate holder.
- For fully skirted plates the MixMate® should be operated without the plate holder. Please see the MixMate® Operating Manual for further instructions.

Manual vortexing

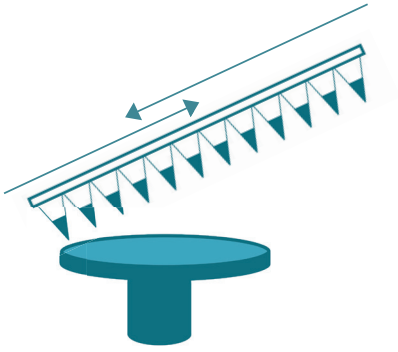
A manual vortex is needed to vortex all separate tubes throughout the protocol. A manual vortex may also be used for the vortexing of plates during the detection step, if so, follow the below instructions. You can also see our vortexing video for further guidance on maual vortexing.

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

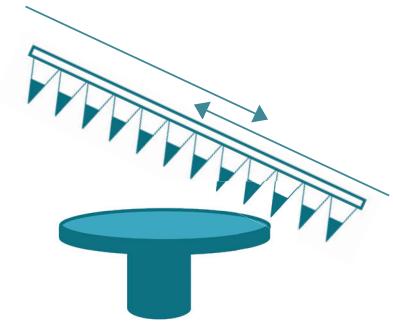
1. Move the plate back and forth over the vortex in horizontal direction. Make sure that the wells at the outside edges of the plate (columns 1 and 12) are also vortexed.



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.

5.3.2 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

It is important to calculate how many samples, replicates and controls that are needed to get the data you want from the study. When running more than one plate, Olink® always recommends randomization of samples as good laboratory practice for all projects. Olink® Focus utilizes three calibrators to determine the protein concentration and randomization of samples is recommended but not mandatory. Randomization refers to the random assignment of samples to all plates and wells included in the study.

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 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 only intended for the two plates used for one Focus IFC.

6.3 Create program

Create a PEA program on the thermal cycler with the following settings.
Heated cover temperature should be 105 °C, and the volume should be set to 100 µl.

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

6.4 Time indications and limits

Step	Duration	Comment
Immuno setup	30–45 min	
Immuno reaction	16–22 hours (overnight)	Keep incubation times consistent when running multiple IFCs for the same project (variation < 2 hours).
Extension	2 hours (30 min hands-on time)	From preparing mix to start of PCR machine and running the PCR program. **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.*
Load/qPCR	2 hours 10 min	

6.5 Sample preparation

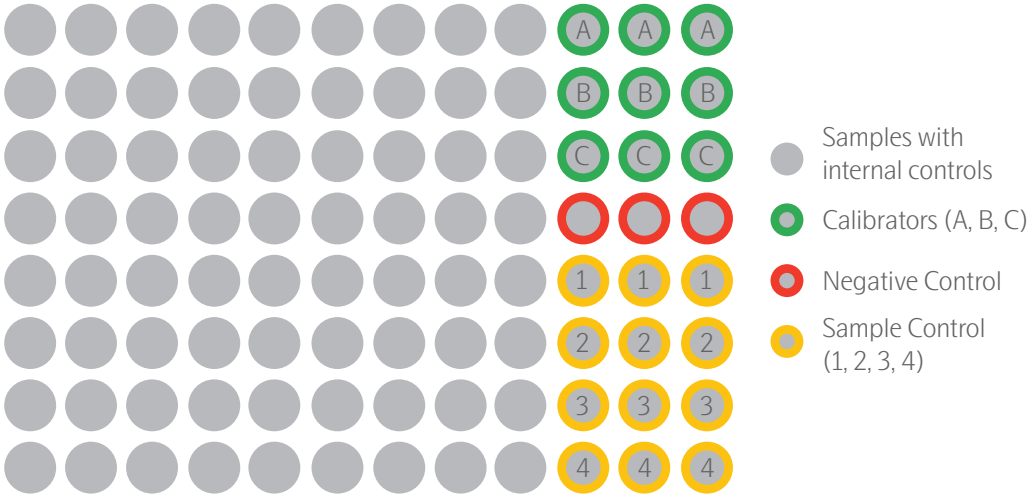
Sample preparation recommendations are listed below:

- Use a 96-well PCR plate format, preferably with full skirt. All plates need to be able to withstand -80 °C and be 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.

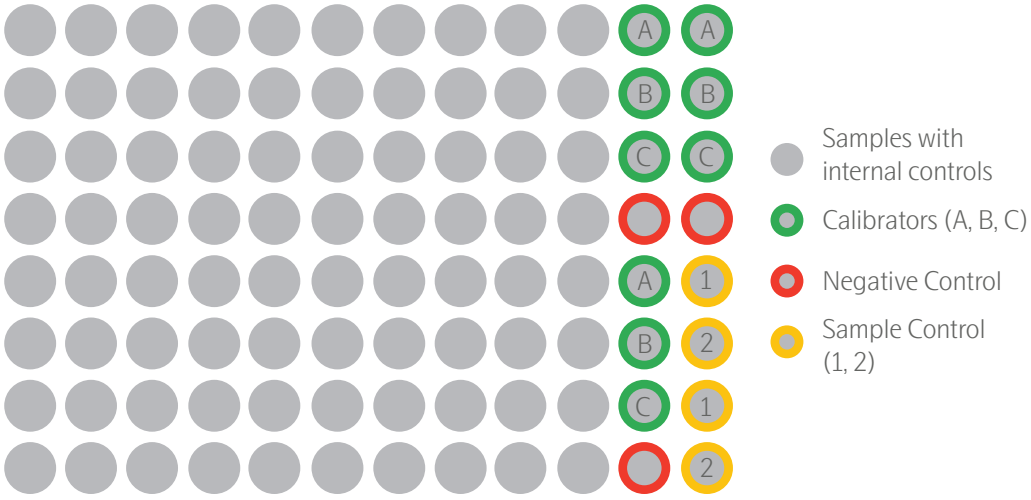
6.6 Plate layout

Below are two illustrations of different plate layouts. The layout might vary between different Focus panels. Always refer to the panel-specific instructions. The controls and calibrators are always included in the kit delivered by Olink.

This is the standard layout for Absolute Quantification panels where columns 10 to 12 consist of controls and calibrators:



This is the standard layout for Relative Quantification panels where columns 11 to 12 consist of controls and calibrators:



7. Dilution step

7.1 Overview

For some Focus panels, a sample dilution step is required prior to running the assay protocol. For Focus 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.*

7.2 Sample dilution step for 1:10 panels

7.2.1 Dilution step instruction for two plates

Prepare bench

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

- 2 prepared 96-well plates with samples
- 2 (two) 96-well PCR plates
- 1 Sample Diluent
- 1 multichannel pipette reservoir (minimum 15 mL)
- 1 multichannel pipette (10 µL)
- 200 multichannel pipette tips (10 µL)
- 4 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 Calibrator samples should **not** be diluted. However, Sample Controls should be diluted.*

- Sample dilutions should be made in two 96-well plates (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.

Instruction

1. Thaw the Sample Diluent, vortex and empty the bottle into a multichannel pipette reservoir.
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 in the sample diluent to pre-condition the pipette tips.
4. Transfer 9 µL of the Sample Diluent to the bottom of all wells except the Negative Control and Calibrator wells on both Dilution Plate 1 and Dilution Plate 2, using **reverse pipetting**. The layout might differ for some Focus panels

and is then stated in the panel-specific instructions. 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 Plates using the MixMate® at 2000 for 30 seconds and spin down the liquid at 400–1000 x g, for 1 minute at room temperature.
6. Carefully transfer 1 µL of your samples and Sample Controls according to your plate layout to the surface of the diluent on the two Dilution Plates using **forward pipetting**. Prepare one plate at a time and carefully check the labeling of the plates to prevent mix-ups.

NOTE: *Use the same multichannel pipette throughout both plates, and also for the Sample Control wells in column 12. Change tips between each pipetting step..*

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

7.2.2 Dilution step instruction for one plate

Prepare bench

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

- 1 prepared 96-well plate with samples
- 1 (one) 96-well PCR plate
- 1 Sample Diluent
- 1 multichannel pipette reservoir (minimum 15 mL)
- 1 multichannel pipette (10 µL)
- 104 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 Calibrator samples should* **not** *be diluted. However, Sample Controls should be diluted.*

- Sample dilutions should be made in one 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.

Instruction

1. Thaw the Sample Diluent, vortex and empty the bottle into a multichannel pipette reservoir.
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 the bottom of all wells except the Negative Control and Calibrator wells on

the Dilution Plate, using **reverse pipetting**. The layout might differ for some Focus panels and is then stated in the panel-specific instructions. 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.
6. Carefully transfer 1 µL of your samples and Sample Controls according to your plate layout to the surface of the diluent on the Dilution Plate using **forward pipetting**.

NOTE: *Use the same multichannel pipette throughout the entire plate, and also for the Sample Control wells in column 12. Change tips between each pipetting step.*

7. Seal both the original Sample Plate and the Dilution Plate with adhesive plastic film.
8. Vortex the Dilution Plate thoroughly using the MixMate® at 2000 rpm for 30 seconds. Refer to *5.3 Vortexing guidelines* for more information.
9. Spin down the content at 400–1000 x g for 1 minute at room temperature.
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.

7.3 Sample dilution step for 1:100 panels

7.3.1 Dilution step instruction for two plates

Prepare bench

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

- 2 prepared 96-well plates with samples
- 2 (two) 96-well PCR plates
- 1 Sample Diluent
- 1 multichannel pipette reservoir (minimum 25 mL)
- 1 multichannel pipette (200 µL)
- 1 multichannel pipette (10 µL)
- 8 multichannel pipette tips (200 µL)
- 200 multichannel pipette tips (10 µL)
- 4 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 Calibrator samples should* **not** *be diluted. However, Sample Controls should be diluted.*

- Sample dilutions should be made in two 96-well plates (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.

Instruction

1. Thaw the Sample Diluent, vortex and empty the bottle into a multichannel pipette reservoir.
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 99 µL of the Sample Diluent to the bottom of all wells except the Negative Control and Calibrator wells on both Dilution Plate 1 and Dilution Plate 2, using **reverse pipetting**. The layout might differ for some Focus panels and is then stated in the panel-specific instructions. 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.
6. Carefully transfer 1 µL of your samples and Sample Controls according to your plate layout to the surface of the diluent on the two Dilution Plates using **forward pipetting**. Prepare one plate at a time and carefully check the labeling of the plates to prevent mix-ups.

NOTE: *Use the same multichannel pipette throughout both plates, and also for the Sample Control wells in column 12. Change tips between each pipetting step.*

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

7.3.2 Dilution step instruction for one plate

Prepare bench

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

- 1 prepared 96-well plate with samples
- 1 (one) 96-well PCR plates
- 1 Sample Diluent
- 1 multichannel pipette reservoir (minimum 15 mL)
- 1 multichannel pipette (200 µL)
- 1 multichannel pipette (10 µL)
- 8 multichannel pipette tips (200 µL)
- 104 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 Calibrator samples should* **not** *be diluted. However, Sample Controls should be diluted.*

- Sample dilutions should be made in one 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.

Instruction

1. Thaw the Sample Diluent, vortex and empty the bottle into a multichannel pipette reservoir (minimum servoir 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 the bottom of all wells except the Negative Control and Calibrator wells on the Dilution Plate, using **reverse pipetting**. The layout might differ for some Focus panels and is then stated in the panel-specific instructions. 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.
6. Carefully transfer 1 µL of your samples and Sample Controls according to your plate layout to the surface of the diluent on the Dilution Plate using **forward pipetting**.

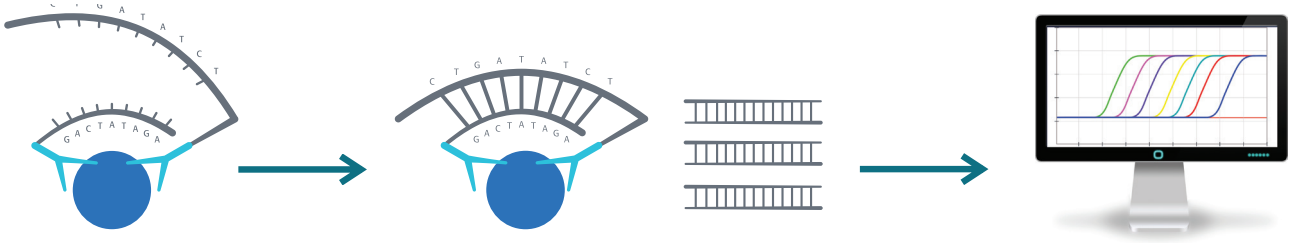
NOTE: *Use the same multichannel pipette throughout the entire plate, and also for the Sample Control wells in column 12. Change tips between each pipetting step.*

7. Seal both the original Sample Plate and the Dilution Plate with adhesive plastic film.
8. Vortex the Dilution Plate using the MixMate® thoroughly at 2000 rpm for 30 seconds. Refer to *5.3 Vortexing guidelines* for more information.
9. Spin down the content at 400–1000 x g for 1 minute at room temperature.
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.

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.



1. Incubation reaction

Duration: Overnight 16–22 hours

Location: Pre-PCR room

Needed for this step:

- Frw-probes
- Rev-probes
- Incubation Solution
- Sample Controls
- Negative Control
- Calibrators

2. Extension and amplification

Duration: 2 hours

Location: Pre-PCR and PCR room

Needed for this step:

- PEA Solution
- PEA Enzyme
- PEA Enhancer
- High Purity Water

3. Detection

Duration: 2 hours 10 min

Location: PCR room

Needed for this step:

- Detection Solution
- Detection Enzyme
- PCR Polymerase
- High Purity Water
- Primer plate
- Olink® 24.192 IFC for Protein Expression (including chip reagents from Standard BioTools)

IMPORTANT: *The protocol described in chapter 7 is optimized for setting up two plates at the time and requires two thermal cyclers. If working with only one thermal cycler, the incubation (8.4 Incubation instruction) can be performed as described but the extension (8.5 Extension and amplification step) have to be performed in two steps (8.5.2 Extension and amplification step instruction for one plate). We recommend that the incubation time within a project should not differ more than 2 hours between plates.*

IMPORTANT: *To ensure that the data produced in your run is reliable, do not leave any sample or primer inlets in the IFC empty. It could cause air to enter the IFC and affect the results of both the empty well, and those surrounding it. Add all mixes without sample, following the protocol. Do not add pure water directly to the IFC inlets. If there isn't enough volume of a primer, please contact support@olink.com for guidance and possible replacement.*

8.2 Incubation reaction step

The Incubation reaction 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.

8.3 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 reaction step, you will need:

- Incubation Solution
- Sample Controls
- Frw- and Rev-probes
- Negative Control
- Calibrators
- Two 8-well strips
- Two prepared 96-well plates with samples in columns 1–9 (1–10 for some Focus panels)
- Two 96-well plates
- 1 microcentrifuge tube (1–1.5 mL)
- 3 pipette tips (10 µL)
- 3 pipette tips (100/200 µL)
- 1 pipette tip (1000 µL)
- 152–176 multichannel pipette tips (10 µL) depending on kit composition
- 4 adhesive films

Vortex and spin all reagents except enzymes before use.

8.4 Incubation instruction

TIP: *Practice reverse pipetting before you start.*

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

1. Thaw the samples, Sample Controls, Negative Control and Calibrators at room temperature. Vortex all samples and reagents and spin briefly.
2. Add at least 12 µL of the three calibrators, four Sample Controls and Negative Control to an 8-well strip according to the following structure. This layout might differ for some Focus panels and is then stated in the panel-specific instructions.

Calibrators A, B, C

Negative control

Sample Control
1, 2, 3, 4

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. Pipette the Incubation Solution carefully to avoid foaming.

Incubation Mix	Per two 96-well plates (µL)
Olink® Focus Incubation Solution	638.0
Olink® Focus Frw-probes	80.0
Olink® Focus Rev-probes	80.0
Total	798.0

4. Vortex and spin down the Incubation Mix. Transfer 92 µL of the Incubation Mix to each well of a new 8-well strip.
5. Pre-condition the multichannel pipette tips and transfer 3 µL of Incubation Mix to the bottom of the wells of column 1–12 in two new 96-well plates by reverse pipetting and name them Incubation Plate 1 and Incubation Plate 2. Use reverse pipetting. The same pipette tips can be used for both entire plates, but if the dead volume starts to look uneven, change pipette tips between columns. Pipette near the surface of the Incubation Mix to prevent liquid from sticking to the outside of the pipette tips.
6. Vortex the plate of samples using MixMate® at 2000 rpm for 30 seconds and spin down the liquid at 400–1000 x g, for 1 minute at room temperature. If the samples are located in tubes, first transfer the samples to a sample plate.
   Transfer 1 µL of each sample, using a multichannel pipette, to the bottom of the wells of column 1–9 in the Incubation Plate according to your sample plate layout.

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

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

7. Always refer to the plate layout stated in the panel-specific instructions since it might differ between panels.

   Do one of the following:
   For standard Absolute Quantification panels:
   Use a multichannel pipette to transfer 1 µL of the Calibrators, the Sample Controls and Negative Control from the prepared 8-well strip, to columns 10–12 on both Incubation Plate 1 and Incubation Plate 2, according to the plate layout stated in the panel-specific instructions.
   Use **forward pipetting**.

   For standard Relative Quantification panels:
   Cut the prepared 8-well strip in half. Use a multichannel pipette with only 4 pipette tips to transfer 1 µL of the Calibrators and Negative Control to the correct wells in columns 11–12 on both Incubation Plate 1 and Incubation Plate 2, according to the plate layout stated in the panel-specific instructions. Change the four pipette tips and transfer 1 µL of the Sample Controls, to the correct wells in column 12 on both Incubation Plate 1 and Incubation Plate 2, according to the plate layout stated in the panel-specific instructions.
   Use **forward pipetting**.
8. Seal the two Incubation Plates thoroughly with adhesive plastic films. Inspect the wells for bubbles to ensure that no sample or control was missed. Spin at 400–1000 x g for 1 minute at room temperature.s

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

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.

10. Incubate the two Incubation Plates overnight at +4 °C for 16–22 hours in a refrigerator or cold room where the light is off when the door is closed.

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

11. Prepare a tube of High Purity Water for the extension step the next day. Keep it at +4 °C overnight.
12. Thaw the PEA Solution over night at +4 °C, and place the PEA Enhancer at room temperature over night.

8.5 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 (+4 °C)
- PEA Solution (keep at room temperature for 15 minutes before use)
- PEA Enzyme

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

- PEA Enhancer (at room temperature)
- 1 centrifuge tubes (> 50 mL)
- 1 multichannel pipette reservoir
- 4 pipette tips (1000 µL)
- 8–16 multichannel pipette tips (100/200 µL) depending on 2- or 1-plate setup
- 2 adhesive films

8.5.1 Extension and amplification step instruction for two plates

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. Make sure that no precipitation has occured. If there is precipitate, vortex and spin again.
2. Pre-heat two PCR machines to 50 °C and pause the PEA program (see *6.3 Create program* for more information about the protocol).
3. Spin down the two Incubation Plates at 400–1000 x g for 1 minute at room temperature.
4. Vortex and spin down the PEA Enzyme and PEA Enhancer briefly.
5. Prepare the Extension Mix in a 50 mL tube according to the table. Add the reagents in the specified order, starting with water:

Order	Extension Mix	Per two 96-well plates (µL)
1	High Purity Water (+4 °C)	16 500
2	PEA Enhancer	2200
3	PEA Solution	2200
4	PEA Enzyme	220
	Total	21 120

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

TIME SENSITIVE STEP: *Perform steps 8–12 during 5 minutes.*

8. Start a 5 minute timer and transfer 96 µL of Extension Mix to the upper parts of each of the well walls of column 1–12 in both the Incubation Plate 1 and Incubation Plate 2 using **reverse pipetting**. The same pipette tips can be used throughout both plates, but if the dead volume starts to look uneven, change pipette tips between columns.

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

9. Seal the plates with new adhesive plastic films.

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

10. Use MixMate® to vortex the plates thoroughly at 2000 rpm for 30 seconds to ensure that all wells are mixed before spinning them down.
11. Double check that all wells in the plates contain the same volume. Note any deviations. This information is needed to interpret the data in the statistical analysis step.
12. Take the Incubation Plates to the PCR room.
13. When the five minutes have passed, immediately place the Incubation Plates in the PCR instruments and resume the PCR PEA program. The program takes approximately 1 hour and 30 minutes.

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.*

14. When the PCR PEA program is finished, continue to *8.6 Detection step.*

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

8.5.2 Extension and amplification step instruction for one plate

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. Make sure that no precipitation has occured. If there is precipitate, vortex and spin again.
2. Pre-heat a PCR machine to 50 °C and pause the PEA program (see *6.3 Create program* for more information about the protocol).

3. Spin down the Incubation Plate at 400–1000 x g for 1 minute at room temperature.
4. Vortex and spin down the PEA Enzyme and PEA Enhancer briefly.
5. Prepare the Extension Mix in a 50 mL tube according to the table. Add the reagents in the specified order, starting with water:

Order	Extension Mix	Per 96-well plate (µL)
1	High Purity Water (+4 °C)	8 250
2	PEA Enhancer	1 100
3	PEA Solution	1 100
4	PEA Enzyme	110
	Total	10 560

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

TIME SENSITIVE STEP: *Perform steps 8–12 during a maximum of 5 minutes.*

8. Start a 5 minute timer and transfer 96 µL of Extension Mix to the upper parts of each of the well walls of column 1–12 in the Incubation Plate using **reverse pipetting**. The same pipette tips can be used throughout the plate, but if the dead volume starts to look uneven, change pipette tips between columns.

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

9. Seal the plate with new adhesive plastic film.

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

10. Use MixMate® to vortex the plate thoroughly at 2000 rpm for 30 seconds to ensure that all wells are mixed before spinning them down.
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.
12. Take the Incubation Plate to the PCR room.
13. When the five minutes have passed, immediately place the Incubation Plate in the PCR instrument and resume the PCR PEA program. The program takes approximately 1 hour and 30 minutes.

IMPORTANT: *Proceed with the next plate as soon as possible to keep incubation times between plates as similar as possible. It cannot differ more than 2 hours.*

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.*

14. When the PCR PEA program is finished, continue to *8.6 Detection step.*

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

8.6 Detection step

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

Prepare bench

For the priming of the IFC:

- Olink® 24.192 IFC for Protein Expression
- 1 syringe control line fluid
- 1 syringe actuation fluid
- 1 syringe pressure fluid

For the Detection step:

- Detection Solution
- High purity water
- Detection Enzyme
- PCR Polymerase
- Primer Plate
- Extension products
- One 8-well strip
- Two 96-well plates
- 1 microcentrifuge tube (1-1.5 mL)
- 2 pipette tips (10 µL)
- 4 pipette tips (100/200 µL)
- 2 pipette tips (1000 µL)
- 4 boxes + 40 multichannel pipette tips (10 µL) (A total of ~424 pipette tips)
- 4 adhesive films

8.6.1 Prepare the 24.192 IFC

Before pipetting assays and samples into the 24.192 IFC, prepare it with control line fluid. The illustrations in steps 4 and 5 below show a 96x96 IFC, but the procedures are the same.

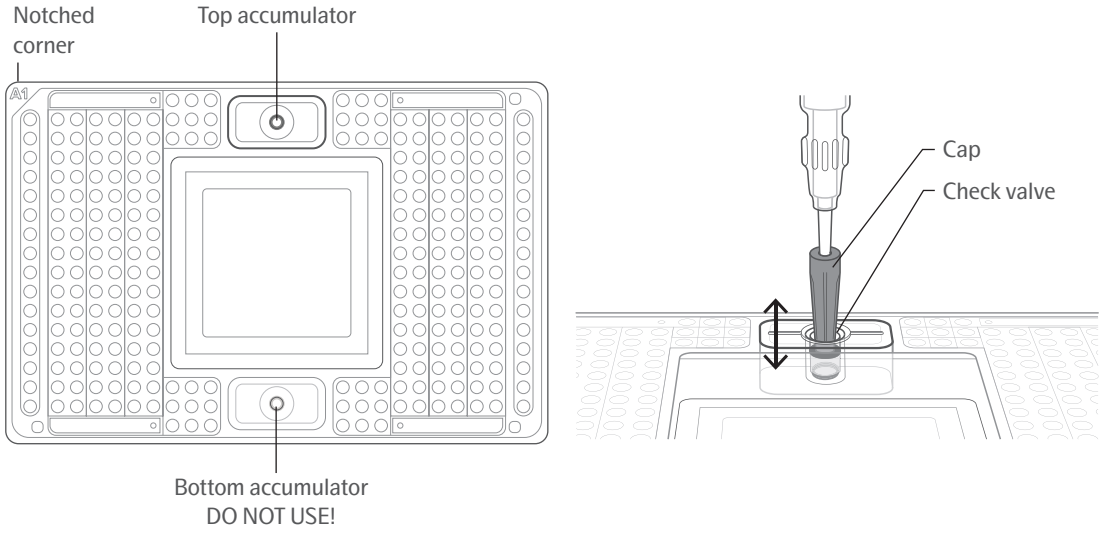
IMPORTANT: *When injecting control line fluid, only use an Olink Control Line Fluid for a 24.192 IFC syringe. Control line fluid syringes are prefilled according to a specific IFC type.*

1. Remove the control line fluid syringe from the packaging and the IFC from the box and foil envelope.

IMPORTANT: *Do not evacuate air from the syringe before injecting control line fluid (Step 4).*

Optional step to ensure proper IFC function:

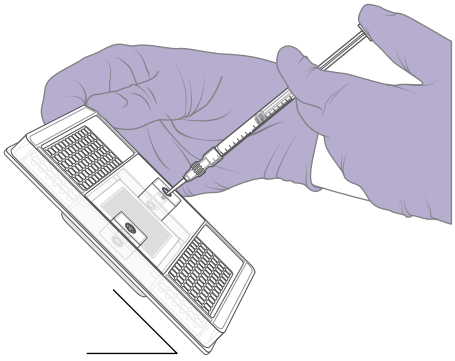
2. Actuate the top check valve:
   a. Place the IFC on a flat surface.
   b. Use the syringe with the shipping cap to actuate the check valve in the top accumulator with gentle pressure. Ensure that the poppet can move freely up and down.



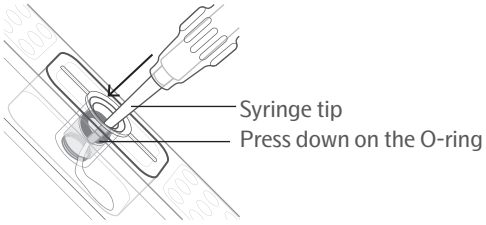
3. Hold the syringe firmly in 1 hand with the tip facing up and away from the IFC, pull back on the plunger slightly to create negative pressure, and remove the shipping cap with the other hand. Be careful when removing the syringe cap of the control line fluid to prevent drips.
4. Holding the IFC at a 45° angle, insert the syringe tip into the top accumulator.

IMPORTANT:

- **Avoid bending the syringe tip. Be careful when removing the syringe cap to prevent drips.**
- **Avoid getting control line fluid on the exterior of the IFC or in the inlets because this makes the IFC unusable. If this occurs, use a new IFC.**



5. Use the syringe tip to press down gently on the black O-ring to move it. Visually confirm that the O-ring has moved.



6. Release the control line fluid:
    a. Press the syringe plunger to release the control line fluid into the accumulator while maintaining the 45° angle to allow the liquid to flow away from the O-ring.
    b. Slowly inject the control line fluid by pushing down on the syringe plunger. The control line fluid flows into the accumulator through the open check valve. Use the entire contents of the syringe.
    c. After fully depressing the plunger, wait approximately 5 sec before withdrawing the syringe. Before removing the

syringe from the accumulator, ensure that all the control line fluid and air are purged from the syringe. This is to avoid dripping fluid on the IFC surface. Residual control line fluid or air in the pipette tip is normal.

7. Make sure that the O-ring returns to its normal position after the syringe is removed.

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 or thaw at room temperature if it has been frozen. 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 in room temperature or refrigerator. 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.

Detection Mix	Per two 96-well plates (µL)
Olink® Focus Detection Solution	690.0
High purity water	285.0
Olink® Focus Detection Enzyme	9.9
Olink® Focus PCR Polymerase	3.8
Total	989.0

5. Vortex the Detection Mix and spin briefly. Transfer 122 µL of the mix to each well of an 8-well strip, using **reverse pipetting**.
6. Use a multichannel pipette to transfer 4.3 µL of the Detection Mix to each well of column 1–12 in two new 96-well plates by **reverse pipetting**. The same pipette tips can be used for both entire plates, but if the dead volume starts to look uneven, change pipette tips between columns. Name these plates Sample Plate 1 and Sample Plate 2.
7. Carefully remove the adhesive film from the Incubation Plates.
8. Transfer 1.7 µL from the extension products in Incubation Plate 1 to Sample Plate 1 and Incubation Plate 2 to Sample Plate 2 using a multichannel pipette and **forward pipetting**. Change tips between each column.
9. Seal all plates with adhesive plastic film.

TIP: *The plate with extension products can be saved up to one week at +4 ºC or up to four weeks at -20 °C in a refrigerator or freezer where the light is off when the door is closed.*

10. Vortex the Sample Plates and Primer Plate and spin them at 400–1000 x g, for 1 minute at room temperature.
11. Double check that all wells in the Sample Plates and Primer Plate contain the same volume. Note any deviations. This information is needed to interpret the data in the statistical analysis step.

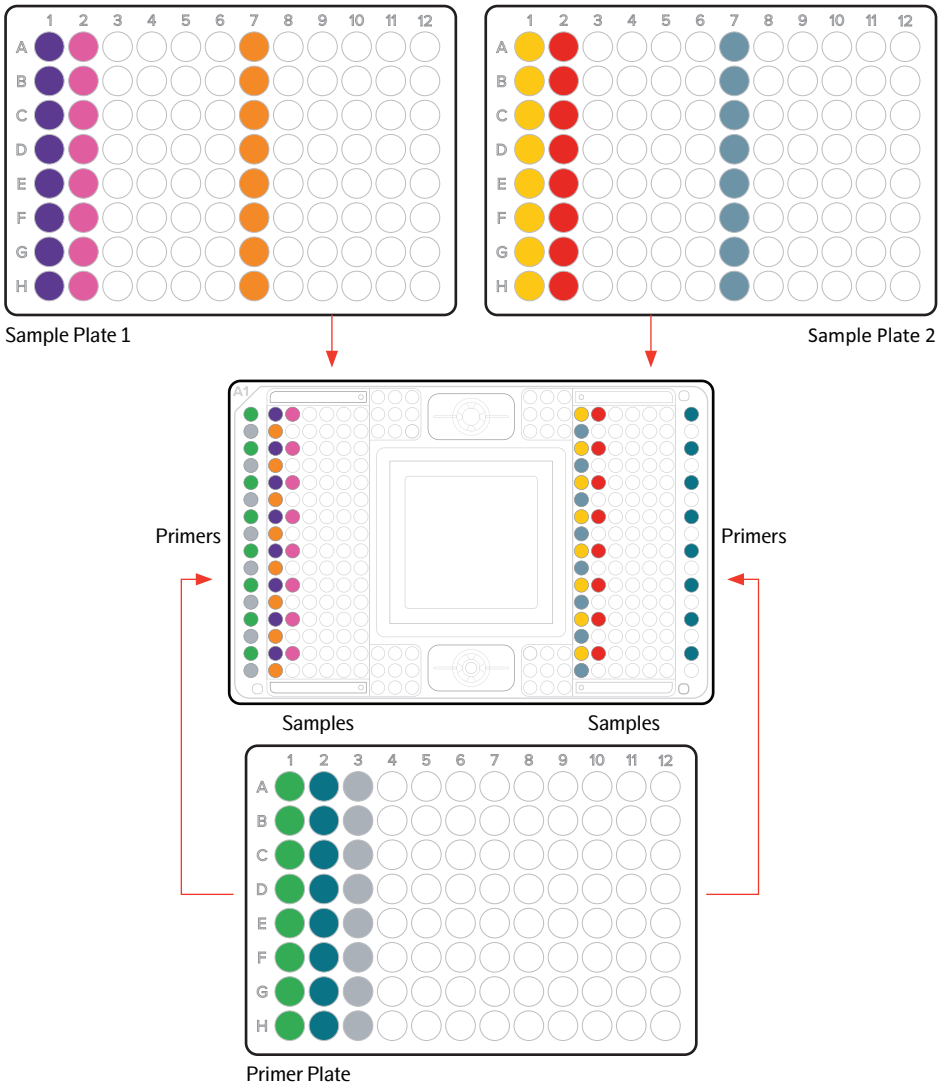
NOTE: *The IFC should be oriented so that the notched corner of the IFC is placed on the upper left side. In the following steps, Sample Plate 1 will be loaded to the left, and Sample Plate 2 to the right. Primers will be loaded in the primer columns on the* 24.192 IFC. *In the next section there is an overview illustration of the loading.*

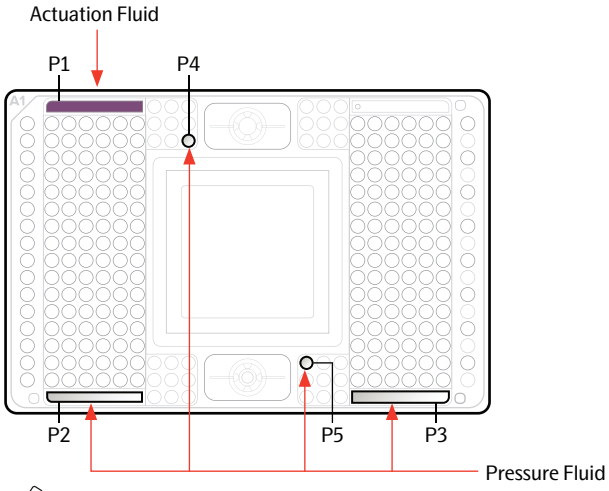
8.6.2 Pipet Primers, Samples, and IFC Reagents into the 24.192 IFC

IMPORTANT:

- **Vortex all Primer Plates, Sample Plates and IFC Reagents thoroughly, then centrifuge them to collect contents before removing the plate seals and pipetting the liquids into the IFC inlets. Failure to do so may result in a decrease in data quality.**
- **While pipetting, do not go past the first stop on the pipette. Doing so may introduce airbubbles into inlets.**

Refer to these figures when pipetting the final samples and primers and when pipetting the IFC reagents (Actuation Fluid, Pressure Fluid) into the IFC. Note the orientation of the A1 corner. The barcoded edge is on the left side.





NOTE: *Olink recommends using the reverse pipetting method when pipetting the samples and primers. See the Olink® Target 96 User Manual (Olink 0935) or the Olink® Target 48 User Manual (Olink 1141).*

1. Thaw the Pressure Fluid at room temperature or in refrigerator.

NOTE: *The Actuation Fluid is in liquid form at -20 °C and stored in the freezer until used.*

2. Pull the protective film down and away from the bottom of the IFC. Discard the film.
3. Pipet 3 µL of each sample into the respective sample inlets on the IFC.
4. Pipet 3 µL of each primer into the respective primer inlets on the IFC.
5. Pipet 150 µL of Actuation Fluid (100–6250) into the P1 reservoir ( ) on the IFC.
6. Pipet 150 µL of Pressure Fluid (100–6249) into each of the P2 and P3 reservoirs ( ) on the IFC.
7. Pipet 20 µL of Pressure Fluid into each of the P4 and P5 inlets ( ) on the IFC.
8. Remove any bubbles and then place the Olink Signature Q100 Interface Plate (24.192) over the IFC, aligning the barcoded edges.
9. Use clear tape to remove any dust particles or debris from the IFC surface, if necessary.

IMPORTANT: *Run the IFC on Olink® Signature Q100 within 60 min of pipetting the samples, primers, and fluids.*

10. On the **Home** screen of Olink Signature Q100, tap **Focus**. Place the prepared IFC 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 Start run screen, tap **Start**. The screen shows the remaining time. For further details, refer to the Olink Signature Q100 User Manual.

9. Standard Biotools™ Biomark™ system

The Olink® Focus panels can be run both on the Olink® Signature Q100 instrument as well as on the Standard Biotools™ Biomark™ system. If using the Standard Biotools Biomark system the additional requirements and operational steps of this chapter should be followed.

9.1 Additional requirements

9.1.1 Analysis software

The Biomark Data Collection software from Standard Biotools needs to be installed on the Biomark computer, and the Real-time PCR Analysis software is required for the analysis of Olink data in the Olink® NPX Signature software. A csv file exported from the Real-time PCR analysis software is needed to be able to analyze Biomark runs in Olink® NPX Signature.

9.1.2 Protein list

The list of proteins can directly be imported into the Standard Biotools analysis software as a .plt file. The relevant .plt file is provided by Olink.

9.2 Preparation — Create programs

9.2.1 Olink® Protein Expression 24x192 program

Create a Biomark system program named "Olink Protein Expression 24×192" 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 (40 cycles)	95°C	15 s
	60°C	60 s

9.3 Operation

9.3.1 Detection step

1. Place the IFC with its barcoding facing you in the IFC Controller. Select **Load Chip** or **LOAD** and the script to run, followed by **Run Script** or **RUN** to load the assay and sample mixes into the central part of the IFC. The loading program takes approximately 35 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. Remove the protective film from the IFC.
4. Load the IFC in the Biomark with the barcode facing outwards and start the Olink Protein Expression protocol. 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.

NOTE: *This video features our 96-plex panels, but most steps are the same for these protocols.*

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
1.4	2023-11-17	Included Chapter 7 Dilution step.
1.3	2023-09-11	Changed 4. Additional requirements. Updated 7.1. Added 7.5.2 Extension and amplification step instruction for one plate. Changed the number of pipette tips needed. Editorial changes.
1.2	2022-12-15	Changed the centrifugation speed for MixMate® to 2000 rpm. Changed last page footer.
1.1	2022-11-04	New control kit box labels for Focus AbsQ. Moved 8.2.2 to chapter 6. Editorial changes.

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1231, v1.4, 2023-11-17