



# Olink® Flex

## User Manual

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# 1. Introduction

## 1.1 Intended use

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

The Olink® Flex panels enable rapid analysis with minimal sample consumption and exceptional data quality, with protein concentrations reported in standard units (pg/mL) as default and optionally in relative (NPX) units. Using just 1 µl of sample, 15–21 proteins are assayed simultaneously on each panel, and results are generated within 24 hours.

## 1.2 About this manual

The Olink® Flex User Manual provides you with the instructions needed to run an Olink® Flex panel using Olink® Signature Q100. If using the BioMark™ system from Standard Biotech®, refer to [8. The BioMark system from Standard Biotech®](#).

### 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® Flex panels have been validated using EDTA plasma and serum samples. Different sample matrices are expected to affect the detection of specific proteins in different ways. For example, extreme levels of IgG or fluorescent particles can interfere with the Olink assay. For more information on sample types, please find the Data Validation document for Olink® Flex on the panel page on our website, or contact Olink support at [support@olink.com](mailto: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](http://www.olink.com/downloads).

#### **User Manuals**

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

#### **White papers**

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

#### **Material Safety Data Sheet**

- Olink® Flex Detection kit
- Olink® Flex Control kit
- Olink® Flex Probe kit

### 1.4.2 Other documents

For information on the Juno and BioMark Systems from Standard Biotech®, read the following User Guides that can be found on [www.standardbio.com](http://www.standardbio.com):

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

For information on the MixMate® read the Operating Manual that can be found on [www.eppendorf.com](http://www.eppendorf.com):

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

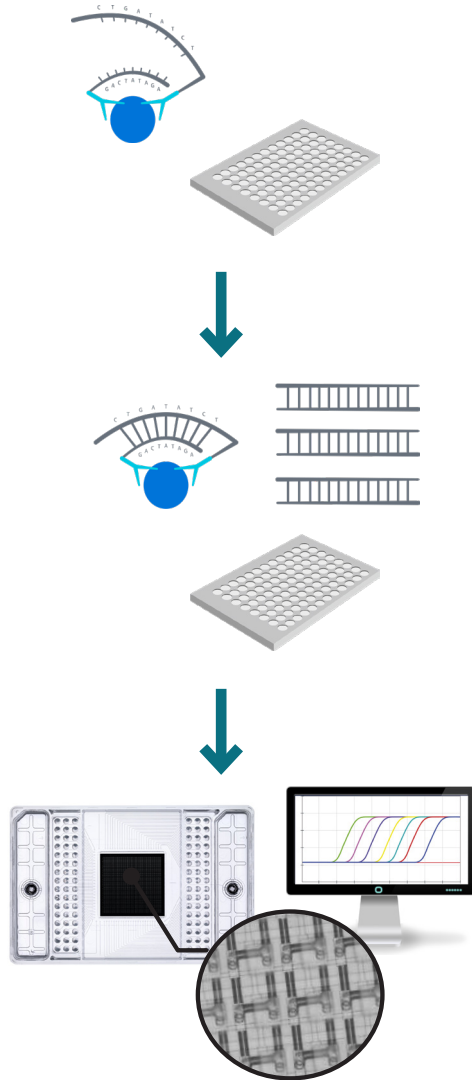
## 1.5 Technical support

For technical support, please contact Olink Proteomics at [support@olink.com](mailto: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 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: 3 hours

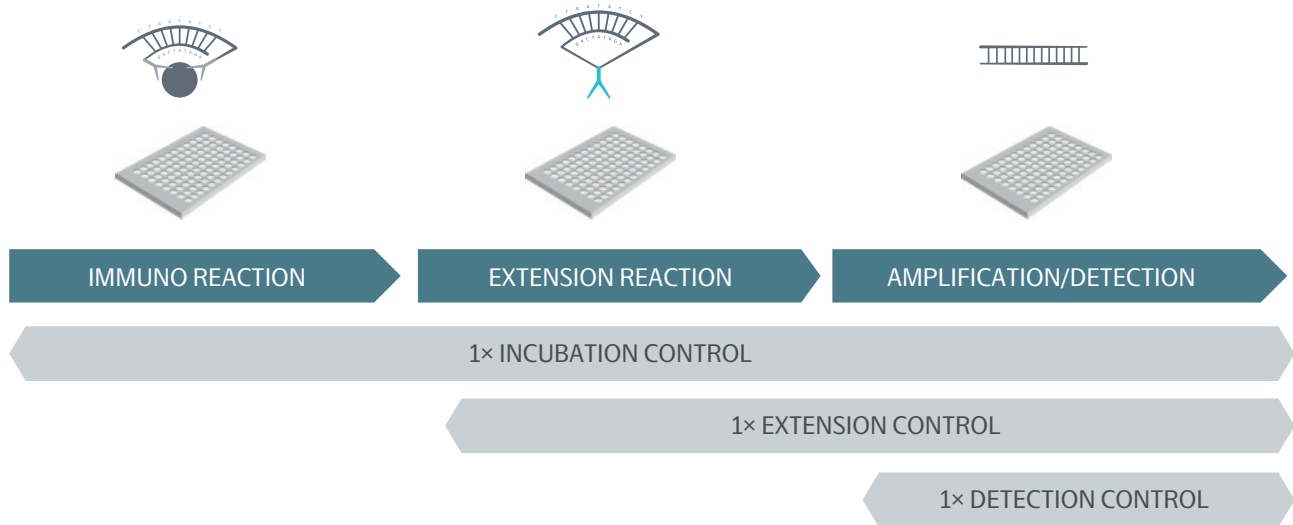
The amount of each DNA barcode is quantified by microfluidic qPCR.

### 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.



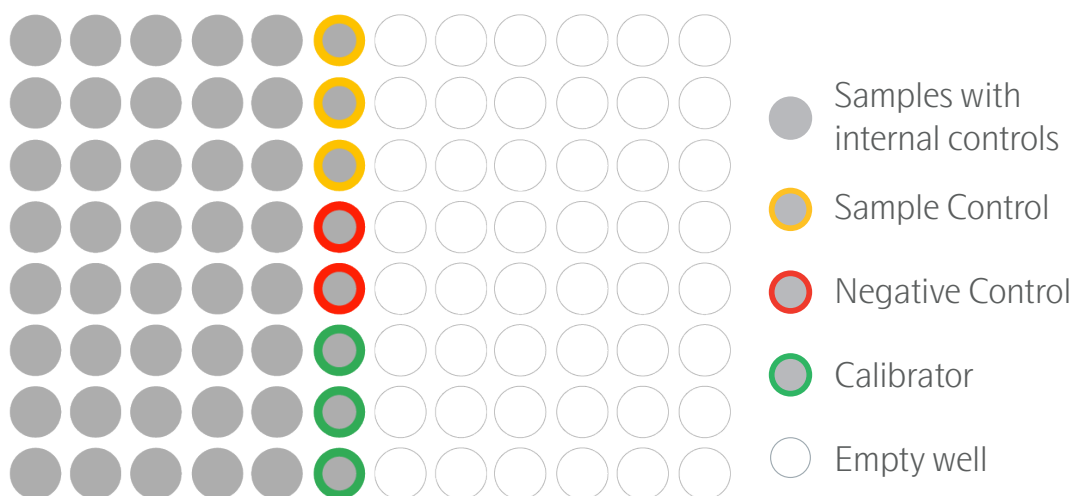
**Incubation control:** The Incubation Control is a non-human antigen measured with PEA. This control monitors potential technical variation in all three steps of the reaction.

**Extension control:** The Extension Control is composed of an antibody coupled to a unique pair of 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.

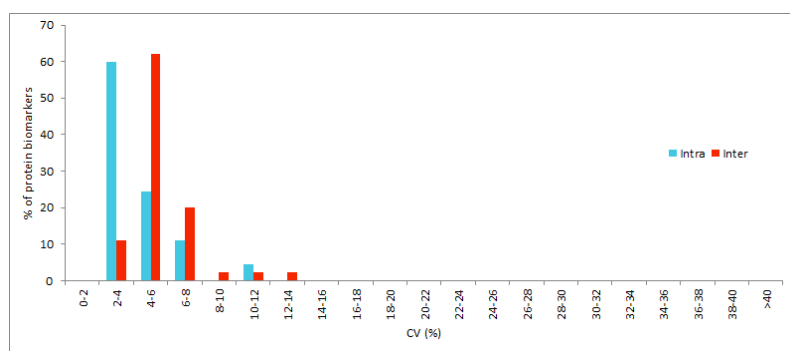
**Detection control:** The Detection Control is a complete double stranded DNA amplicon which does not require any proximity binding or extension step to generate a signal. This control monitors the amplification/detection step.

### 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 40 samples, three Sample controls, two Negative controls and three Calibrators.



**Sample Control:** The Sample Control is a pooled human plasma sample, with spiked in recombinant antigens for some proteins to ensure that all proteins in the panel are detected, and included in triplicate on each plate. The Sample Control is used to assess potential variation between runs and plates, as well as quality control of the quantification of data in pg/mL. See the Quantified data section below. Validation data for all panels is available on the Olink website. An example of inter- and intra-assay CV is illustrated here:



**Negative Control:** Negative Control is included in duplicate 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, defined as three fixed standard deviations above average for the negative controls.

**Calibrator:** The Calibrator is a pooled human plasma sample with spiked in recombinant antigens for proteins with low endogenous levels to ensure that all proteins are detected within the limits of quantification (LOQ). The calibrator is used to adjust the predefined standard curve along the y-axis during development at Olink. The calibrator is also included in triplicate on each sample plate and used to normalize all samples. See the Quantified data section below for details.

### 2.2.3 Data analysis

Protein expression is reported in standard concentration units (pg/mL) as default. Optionally, it can also be reported in relative Normalized Protein eXpression (NPX) units. 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). 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 Olink Flex panels:

Each sample is normalized with its corresponding extension control level:

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

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

$$\text{Bridging factor}_{\text{Assay } j} - (dCt_{\text{Sample } i, \text{ Assay } j} - \text{Median}(\text{Calibrator}_{\text{Sample } i, \text{ Assay } j})) = \text{NPX}_{\text{Sample } i, \text{ Assay } j}$$



**NOTE:** Because NPX is a relative unit, the NPX value will differ between the same protein measured with different panels regardless if it is a comparison between for example two different Target 96 panels or a Flex and a Target 48 panel. However, the ratios between samples should remain the same between different panels.

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 Olink Flex is described in this section.

### Method overview

The method requires fewer sample wells on each plate/run for controls compared to many other platforms, making sure that as many wells as possible can be used for customer samples.

### Before run (during product development at Olink)

1. A precise pre-defined standard curve is established for each protein.
2. The triplicate calibrator is 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.

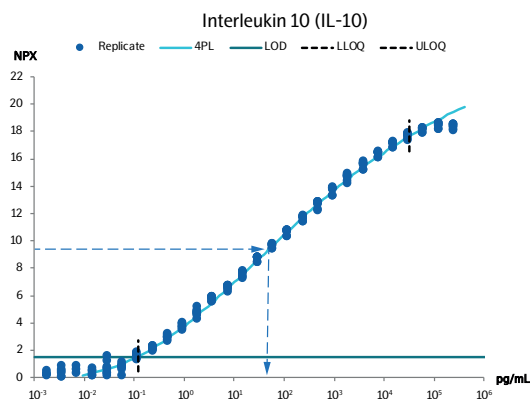
### At each run

1. The median value of the calibrator triplicates 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. 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 are commonly used in many technologies, would not suffice for Olink's multiplex measurements. The predefined standard curves avoid operator dependent reconstitution and pipetting of standard curves at each lab and for each run. The following figure shows an example of a point standard curve defined for each assay during development. Standard curves for each assay can be found via the panel product page ([www.olink.com/flex](http://www.olink.com/flex)).





### 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 limits of quantification (LOQ). The lower and upper limits of quantification (LLOQ and ULOQ) are defined during the development of the panel, see the panel specific Validation data document available on the Olink website ([www.olink.com](http://www.olink.com)) for details.

### 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 ( $\pm 30\%$  of expected value) and assure the correct functionality of the quantification method.

# 3. Product description

## 3.1 Reagents supplied in Olink kits

Each Olink kit contains reagents for 48 wells, sufficient for 40 samples and 8 controls. 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 Flex kits are lot specific and reagents from different kit lots cannot be combined.

### 3.1.1 Probe kit

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

Part	Description
Olink® 1-48 plex Incubation Solution	Contains components needed for the immuno reaction
Olink® Flex Frw-probes	Contains antibody probes labeled with Frw-oligos
Olink® Flex Rev-probes	Contains antibody probes labeled with Rev-oligos

Olink® Flex  
Probe Kit

Product no: 93300A  
Lot no:FCFD-MNGF-10-1A  
Expiry date:NOV 2024

Store at:  
+4°C

Box:  
1/3

### 3.1.2 Detection kit

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

Part	Description
Olink® 1-48 plex PEA Solution	Contains components needed for the extension reaction
Olink® 1-48 plex PEA Enzyme	For extension of Frw- and Rev-probes bound to their target
Olink® 1-48 plex PCR Polymerase	For pre-amplification of the extension product created by the PEA Enzyme
Olink® 1-48 plex Detection Solution	Contains components needed for the detection reaction
Olink® 1-48 plex Detection Enzyme	For qPCR amplification
Olink® 1-48 plex Primer Plate	96-well plate with ready-to-use primers for amplification of extension product
Olink® 1-48 plex Negative Control	For determination of background levels
Olink® 1-48 plex PEA Enhancer	Contains components needed for the extension reaction

Olink® Flex  
Detection Kit  
Product no: 93300B  
Lot no: B32104  
Expiry date: NOV 2024

Store at:  
-20°C

Box:  
2/3



### 3.1.3 Control kit

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

Part	Description
Olink® Flex Calibrator	Used for normalization between plates and of samples
Olink® Flex Sample Control	Used for quality control of quantification of data in pg/mL and to assess variation between runs and plates

Olink® Flex  
Control Kit  
Product no: 93300C  
Lot no: B32105  
Expiry date: NOV 2024

Store at:  
-80°C

Box:  
3/3



# 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 for Olink® Flex User Guide 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)

Contact Olink support at [support@olink.com](mailto: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
- Freezer (-80 °C) where the light is off when the door is closed
- Olink Signature Q100
  - alternatively Biomark or Biomark™ HD system from Standard Bioteools®

# 5. Laboratory work safety and guidelines

## 5.1 Safety considerations

### 5.1.1 Safety

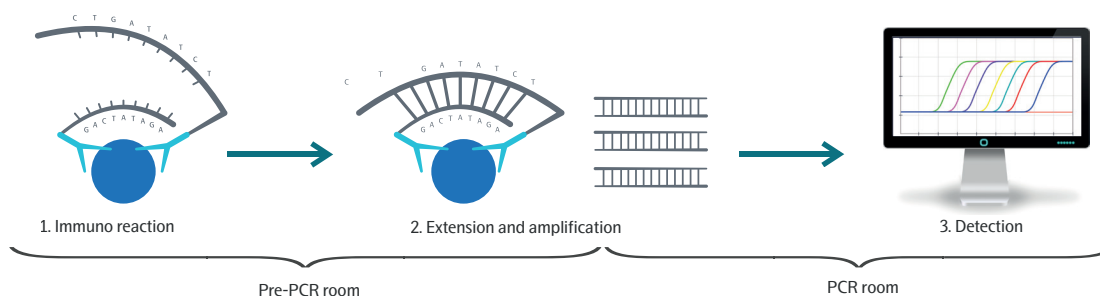
Follow general laboratory safety procedures:

- 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.
- Handle and dispose of hazardous sample material according to local regulations.

For complete safety information, refer to Safety Data Sheets (SDS) available on the Olink website: [www.olink.com/downloads](http://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 Immuno 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.

Ready position	1	2	3	4
First stop	↓	↑	↓	↑
Second step			↓	

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.

Ready position	1	2	3	4	5	...	X	End
First stop	↓	↑	↓	↑	↓			↑
Second step	↓	↑					↓	↑

## 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.

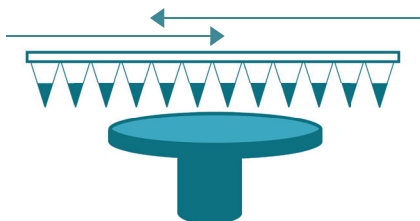
- Set mixing speed at 2000 rpm and mixing time at 30 seconds.
- Start mixing by pressing the start/stop key. After 30 seconds, MixMate® will automatically stop.
- Take out the plate by pulling it out from the front side of the plate holder.
- For fully skirted plates the MixMate® should be operated without the plate holder. Please see the MixMate Operating Manual for further instructions.

### Manual vortexing

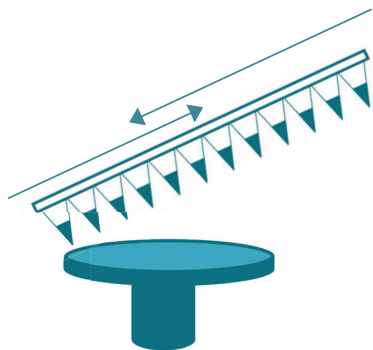
A manual vortex is needed to vortex all separate tubes throughout the protocol. A manual vortex may also be used for the vortexing of plates during the detection step, if so, follow the below instructions. You can also see our vortexing [video](#) for further guidance on manual vortexing.

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

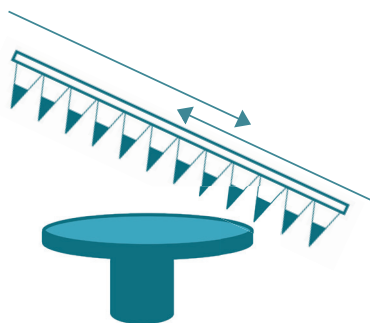
1. Move the plate back and forth over the vortex in horizontal direction. Make sure that the wells at the outside edges of the plate (columns 1 and 12) are also vortexed.



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 Flex utilizes a calibrator 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](mailto: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](http://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 single use only.

## 6.3 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	30 min	From preparing mix to start of PCR machine.  <b>IMPORTANT:</b> 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	10 min	Prepare sample plate during priming of the IFC.
Load	50 min	Start adding primers and samples to the primed IFC within 10 minutes of completed priming . Start the load script in the Olink Signature Q100 within 40 minutes The time in the Signature Olink Q100 is 50 minutes.
qPCR	1.5 hours	Move the loaded IFC from Olink Signature Q100 within 30 minutes of loading completion.  <b>NOTE:</b> We do not recommend setting up more than 3 plates at a time.

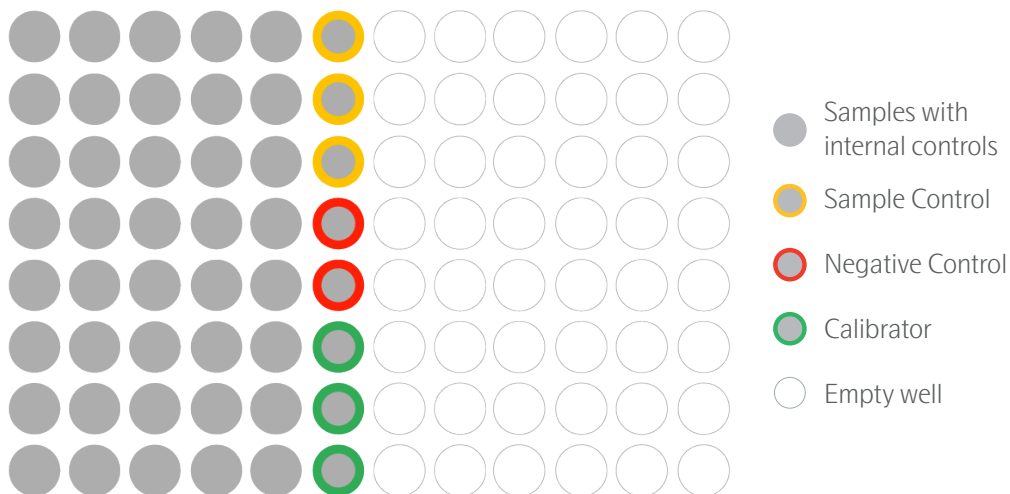
## 6.4 Sample preparation

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.

## 6.5 Plate layout

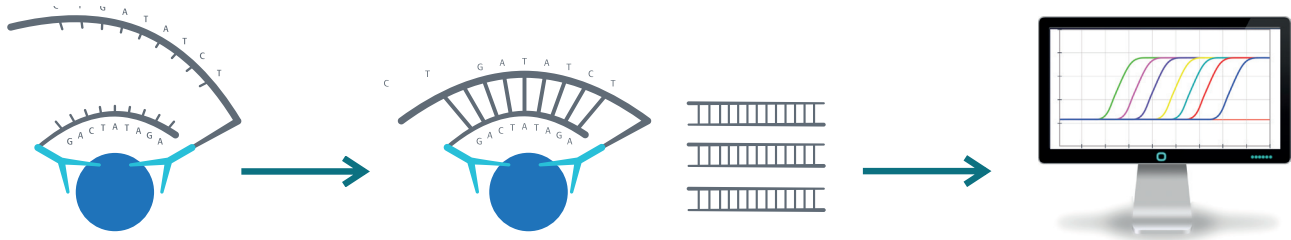
Below is an illustration of the plate layout. The sixth column consists of controls. The controls and calibrator are always included in the kit, and supplied by Olink.



# 7. Operation

## 7.1 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 Control
- Negative Control
- Calibrator

### 2. Extension and amplification

Duration: 2 hours

Location: Pre-PCR and PCR room

Needed for this step:

- PEA Solution
- PEA Enzyme
- PEA Enhancer
- MilliQ

### 3. Detection

Duration: 2.5 hours

Location: PCR room

Needed for this step:

- Detection Solution
- Detection Enzyme
- PCR Polymerase
- Primer plate
- Olink IFC (48.48GT)

## 7.2 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.

## 7.3 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
- Calibrator
- Two 8-well strips
- Prepared 96-well plate with samples in columns 1-5
- 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

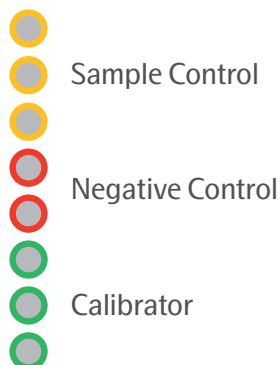
**Vortex and spin all reagents before use.**

## 7.4 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 Control, Negative Control and Calibrator at room temperature. Vortex all tubes and reagents and spin briefly.
2. Add 4 µL of the Calibrator, Sample Control and Negative Control to an 8-well strip according to the following structure:



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 ½ 96-well plate (µL)
Olink® 1-48 plex Incubation Solution	168.0
Olink® Flex Frw-probes	21.0
Olink® Flex Rev-probes	21.0
Total	210.0

4. Vortex and spin down the Incubation Mix. Transfer 23 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 1-6 in a new 96-well plate by reverse pipetting and name it Incubation Plate. Use reverse pipetting and 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 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 samples are located in tubes, use a regular pipette transfer 1 µL of each sample, using a multichannel pipette, to the bottom of the wells of column 1-5 in the Incubation Plate according to your sample plate layout.  
Use **forward pipetting** and change pipette tips between every column.
7. Use a multichannel pipette to transfer 1 µL of Calibrator, Sample Control and Negative Control from the prepared 8-well strip, to column 6 of the Incubation Plate, according to the plate layout shown in step 2.  
Use **forward pipetting**.
8. 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 Plate*

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 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.

**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.


## 7.5 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 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

### 7.5.1 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. Make sure that no precipitation has occurred. If there is precipitate, vortex and spin again.

2. Pre-heat the PCR machine to 50 °C and pause the PEA program.
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 15 mL tube according to the table. Add the reagents in the specified order, starting with water:

Order	Extension Mix	Per ½ 96-well plate (µL)
1	High Purity Water (+4 °C)	4350
2	Olink® 1-48 plex PEA Enhancer	580
3	Olink® 1-48 plex PEA Solution	580
4	Olink® 1-48 plex PEA Enzyme	58
Total		5568


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 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 1-6 in 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.

9. Seal the plate with a 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 it 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.

 **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 (~1 hour 55 minutes), continue to [7.6 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.

## 7.6 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

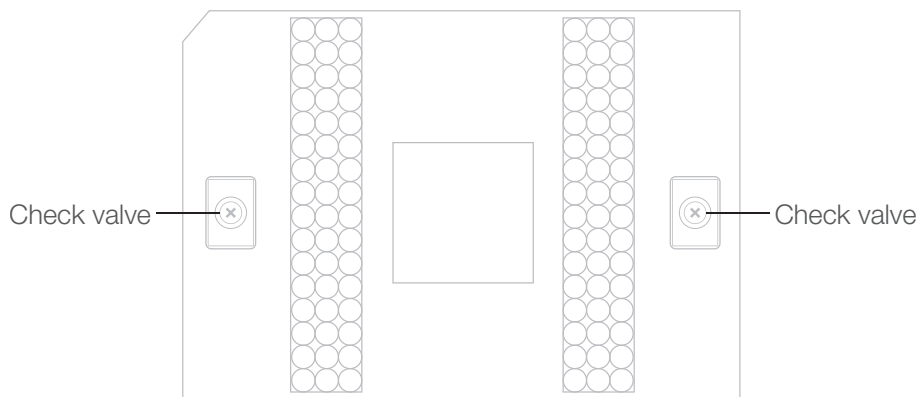
For the priming of the IFC:

- Olink® 48.48 IFC for Protein Expression
- Two syringes with control line fluid for 48.48 IFC

For the Detection step:

- Detection Solution
- High purity water
- Detection Enzyme
- PCR Polymerase
- Primer Plate
- Extension products
- One 8-well strip
- 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)
- 1 box + 8 multichannel pipette tips (10 µL)
- 2 adhesive films

### 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. Make sure that the fluid flows away from the accumulator, otherwise a vacuum is formed and the oil bubbles up.
4. Remove the film from the bottom of the IFC.

- On the **Home** screen of the Signature Olink Q100 instrument, tap **Flex**. Place the chip containing control line fluid and the interface plate on the drawer. Align the notched corner of the chip to the notch on the drawer and face the barcoded edges of the chip 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:** Prepare the Detection Mix during the priming of the IFC.


## Detection step instruction

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


- Remove the Extension products from the PCR instrument. Vortex and spin down the liquid.
- 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.
- 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.
- Prepare the Detection Mix in a microcentrifuge tube. Vortex and spin down the enzymes before adding them to the mix.

Detection Mix	Per ½ 96-well plate (µL)
Olink® 1-48 plex Detection Solution	275.0
High purity water	116.0
Olink® 1-48 plex Detection Enzyme	3.9
Olink® 1-48 plex PCR Polymerase	1.5
Total	396.4

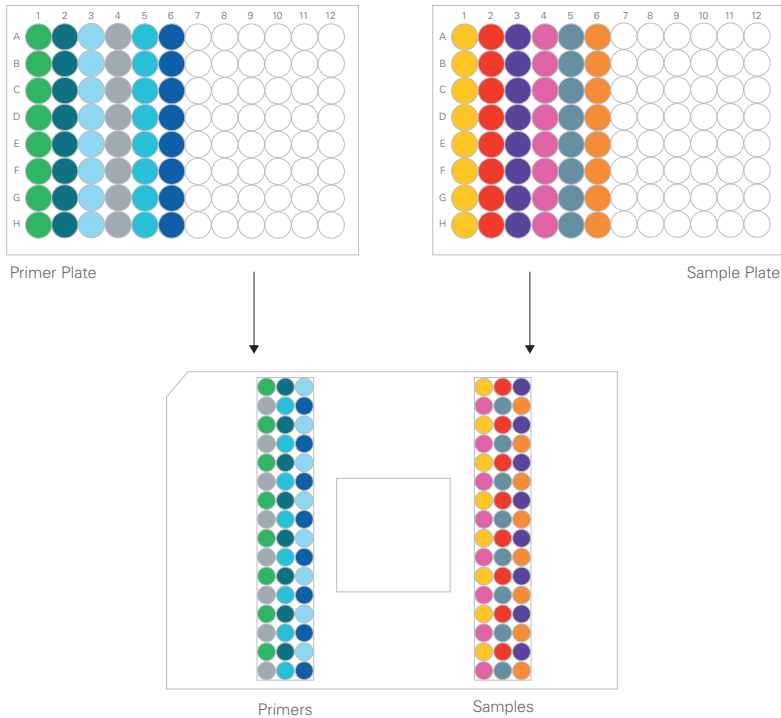
- Vortex the Detection Mix and spin briefly. Transfer 46 µL of the mix to each well of an 8-well strip, using **reverse pipetting**.
- Use a multichannel pipette to transfer 7.2 µL of the Detection Mix to each well of column 1-6 in a new 96-well plate by **reverse pipetting**. Use the same pipette tips throughout the plate. Name this plate Sample Plate.
- Carefully remove the adhesive film from the Incubation Plate.
- 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.
- Seal both the Sample Plate and the plate with extension products with adhesive plastic film.

 **TIP:** The plate with extension products can be saved 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.

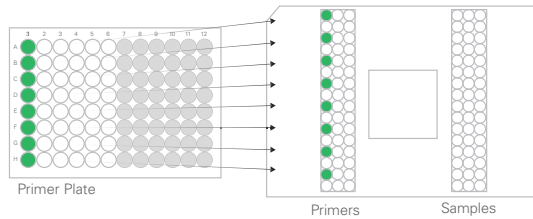
- Vortex the Sample Plate and Primer Plate and spin both at 400-1000 x g, for 1 minute at room temperature.
- Double check that all wells in the Sample Plate and Primer Plate contain the same volume. Note any deviations. This information is needed to interpret the data in the statistical analysis step.
- Remove the primed IFC from the Olink Signature Q100.

 **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 Olink® 48.48 IFC for Protein Expression. Here follows an overview illustration of the loading:

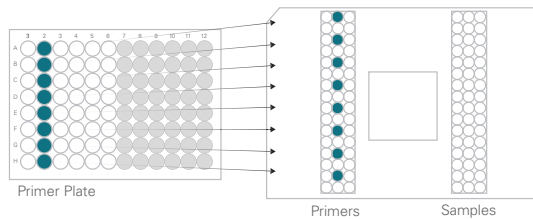




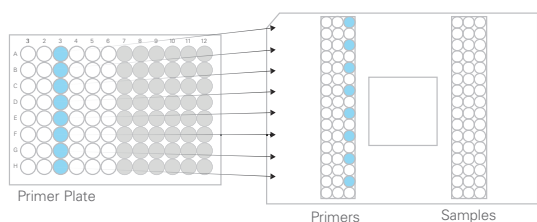
13. Carefully remove the adhesive film from the Primer Plate to avoid contamination between wells.
14. Transfer 5  $\mu\text{L}$  of each primer using **reverse pipetting** from each well in position 1 A-H (green) to the inlets in the first column on the left side of the IFC (green). Change pipette tips after each column. When using an eight-channel pipette every other inlet will be filled according to the image.



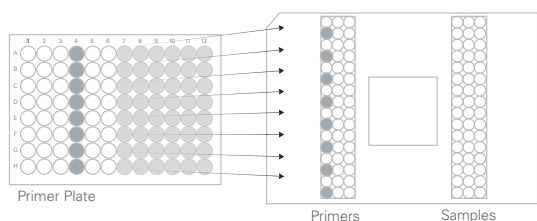
15. Transfer 5  $\mu\text{L}$  from each well in position 2 A-H (teal) to the second column of inlets (teal) according to the image. Use **reverse pipetting** and change tips between each column.



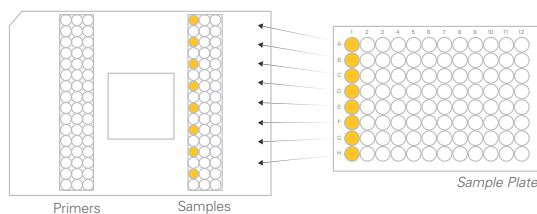
16. Transfer 5  $\mu$ L from each well in position 3 A-H (light blue) to the third column of inlets (light blue) according to the image. Use **reverse pipetting** and change tips between each column.



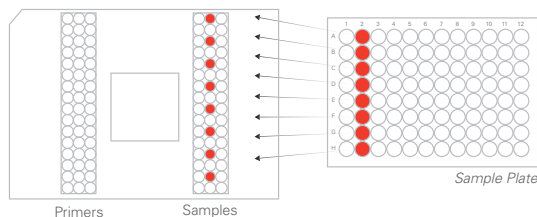
17. Transfer 5  $\mu$ L from each well in position 4 A-H (grey) to the inlets in the first column on the left side of the IFC (grey), start on the second row according to image. Continue with columns 5-6. Use **reverse pipetting** and change tips between each column.



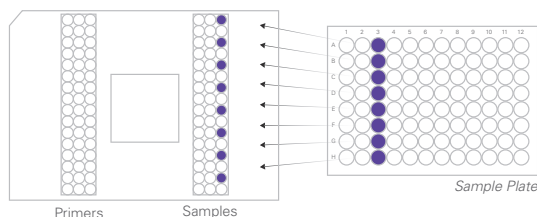
18. Transfer 5  $\mu$ L of each sample using **reverse pipetting** from each well in position 1 A-H (yellow) to the inlets in the first column on the right side of the IFC (yellow). Change pipette tips after each sample. When using an eight-channel pipette every other inlet will be filled according to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



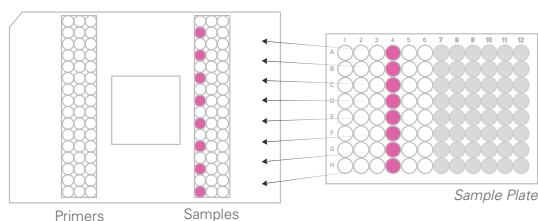
19. Transfer 5  $\mu$ L from each well in position 2 A-H (red) to the second column of inlets (red) according to the image. Use **reverse pipetting** and change tips between each column.



20. Transfer 5  $\mu$ L from each well in position 3 A-H (purple) to the second column of inlets (purple) according to the image. Use **reverse pipetting** and change tips between each column.



21. Transfer 5  $\mu$ L from each well in position 4 A-H (pink) to the inlets in the first column on the right side of the IFC (pink), start on the second row according to image. Continue with columns 5-6. Use **reverse pipetting** and change tips between each column.



22. When loading is finished, inspect the wells and remove any bubbles using a syringe needle. Change needle between wells to avoid contamination.
23. 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.
24. On the Pipette samples and primers onto the chip screen on Olink Signature Q100, tap **Open drawer**. 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 chip and in level with the drawer. Align the notched corner of the chip 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.
25. When the run is complete, remove the IFC and interface plate from the instrument.

# 8. The BioMark system from Standard Biotools®

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

## 8.1 Additional requirements

### 8.1.1 Analysis software

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

### 8.1.2 Protein list

The list of proteins can directly be imported into the Fluidigm analysis software as a .plt file. The relevant .plt file can be found on the lot configuration data sheet provided in every kit.

Download the Detector Setup.plt file corresponding to your panel at [www.olink.com/members-site](http://www.olink.com/members-site).

## 8.2 Preparation - Prepare files

### 8.2.1 Enable script on Juno System for loading of 48.48 GT IFCs

To load the Olink® 48.48 IFC for Protein Expression on the Juno System, the correct script must be enabled.



**NOTE:** The script for loading on the Olink Signature Q100 is enabled by default, 48.48 CS Load Mix.

1. Log in as administrator, tap **Admin** below the tabs (boxes).
2. On the **Tools** screen, tap **Preferences**.
3. Select **Enabled** under **Advanced Scripts**.
4. On the IFC control tab, select **Load Mix 48.48 GT**.

### 8.2.2 Replace definition file

To run the Olink® 48.48 IFC for Protein Expression on the Biomark HD with the Olink program, the definition file needs to be replaced.



**NOTE:** This should not be performed while the Biomark is running a program.

1. Use the Biomark computer to find the IFC definition file M48CS2ChipDefinition.xmx on the Olink Membership Site.
2. Right-click and click **Save as**, and navigate to C:/Program files/Fluidigm/BioMarkDataCollection/ApplicationData/Chip Definitions.
3. Replace the current M48CS2ChipDefinition.xmx file with the M48CS2ChipDefinition.xmx file from the Olink Membership Site.



**NOTE:** This IFC definition file retains all the original functionality of the replaced file. In addition it has added functionality for the Olink Flex assay.

4. Restart the Biomark Data Collection Software.

### 8.2.3 Import the 48x48 Olink Protein Expression 40 cycles program

1. On the Biomark computer, go to **My Computer** and select **C:**.
2. Navigate to *Program files/Fluidigm/BioMarkDataCollection/ApplicationData/Protocols*.
3. Create a new folder, and name it *Olink*.
4. Find the 48x48 Olink Protein Expression 40 cycles.pcl file on the Olink Membership Site. Right-click and click **Save as**.
5. Navigate to *C:/Program files/Fluidigm/BioMarkDataCollection/ApplicationData/Protocols/Olink* and save the 48x48 Olink Protein Expression 40 cycles.pcl file in the *Olink* folder.

### 8.2.4 Create the Olink project setting on the Biomark

1. Open the Biomark Data Collection Software on the Biomark computer.
2. Go to *Settings>Manage Project Settings*.
3. Click **Add New Project Setting** and name the project *48x48 Olink Protein Expression 40 cycles*.
4. Make the following settings:

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

5. Under **Naming and Location, Chip Run Directory**, click **Browse**. Navigate to the folder where you want to save the data files.
6. To upload the protocol, click **Browse for Protocol** under **Thermal Protocol** section and select the 48x48 Olink Protein Expression 40 cycles protocol.
7. **Save** and **Close** and then click **Close** again.
8. Select the **Project Setting** *48x48 Olink Protein Expression 40 cycles* in the Biomark Data Collection starting window



**TIP:** Select the *Remember Project Setting* checkbox to make it the default project setting.

The 48x48 Olink Protein Expression 40 cycles program includes the following steps:

Step	Temperature	Duration
Pre-heat	50°C	120 s
Hot start	95°C	300 s
PCR cycle (40 cycles)	95°C	15 s
	60°C	60 s

## 8.2.5 Olink extension and pre-amplification program settings

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

9. When preparation is complete, tap  (Eject) in the bottom right corner to open the drawer.

Continue to [6.2 Bench setup](#).

## 8.3 Operation

### 8.3.1 Detection step

#### Prime IFC

After removing the film from the bottom of the IFC (step 4), place the IFC, with barcoding facing you, in the IFC Controller. On the touch-screen, first press **Load Chip** or **LOAD** and then select the program *48x48CS Chip Prime* for IFC controller MX and *Prime 48.48 GT* for Juno System. Press **Prime** or **RUN**. The program takes approximately 10 minutes.

Continue to [Detection step instruction](#).

#### Detection step instruction

When finished with step 22, continue with:

1. Place the IFC with its barcoding facing you in the IFC Controller. Select **Load Chip** or **LOAD** and the script to run. Use the *48x48 CS Load mix* for IFC controller MX and *Load Mix 96.96 GE* for Juno System, 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 50 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. Load the IFC in the BioMark™ Reader with the barcode facing outwards and start the 96x96 Olink Protein Expression 40 cycles protocol.

The Biomark program takes approximately 1 hour and 30 minutes.

# 9. Other resources

## 9.1 Olink FAQs

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

## 9.2 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](http://www.olink.com/downloads).

## 9.3 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.

## 9.4 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.

# 10. Revision history

Version	Date	Description
1.0	2023-02-10	First version

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1314, v1.0, 2023-02-10