1. PURPOSE

   To describe the measurement procedure of 92 protein biomarkers in 1 µL sample volume through Proseek® Multiplex$^{96\times96}$ Olink. The Proseek reagents are based on PEA, a Proximity Extension Assay technology, in which 96 oligonucleotide-labeled antibody pairs are allowed to bind to their respective protein targets in the sample. A PCR reporter sequence is formed by a proximity-dependent DNA polymerization event and is subsequently detected and quantified using real-time PCR. The assay is performed in a homogeneous 96-well format with no need for washing steps.

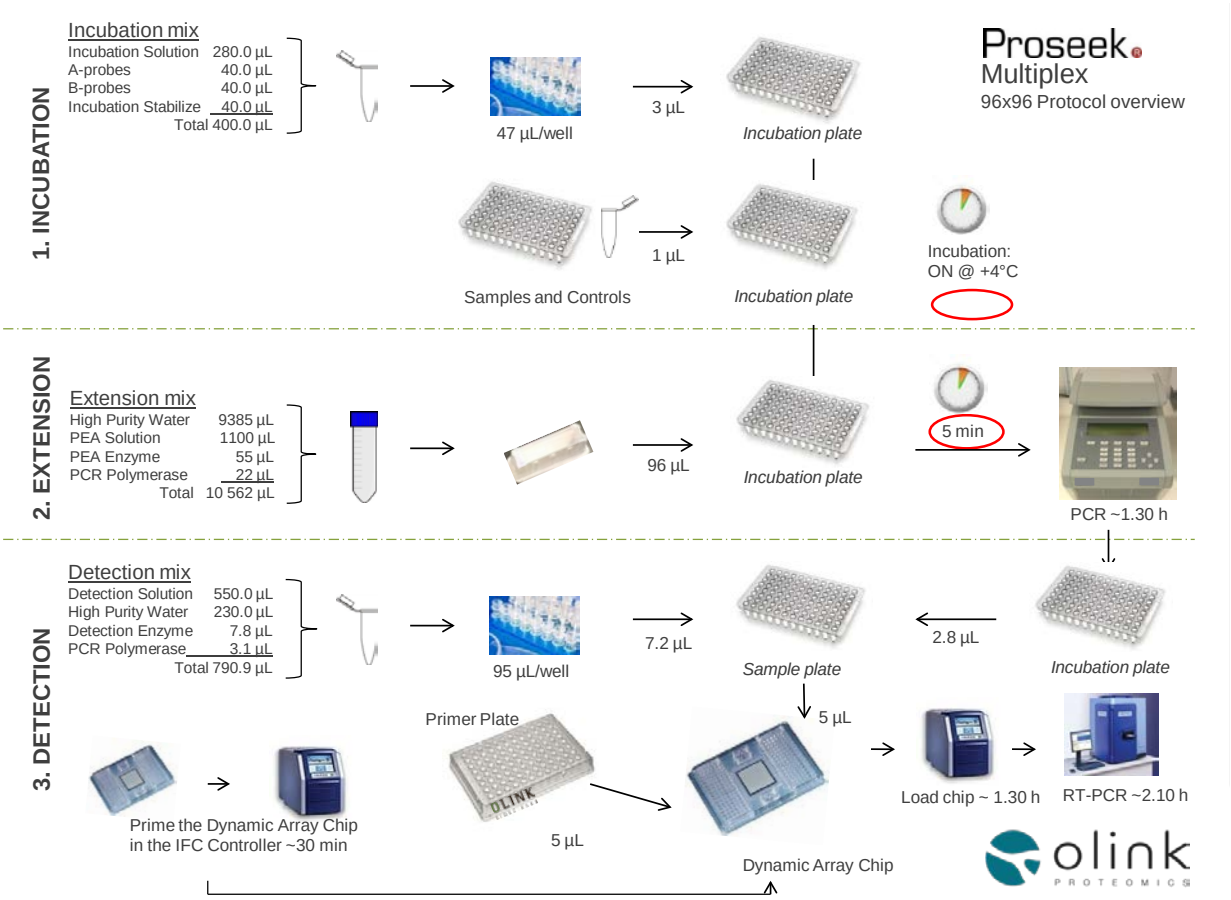
2. REFERENCE-Proseek® Multiplex $^{96\times96}$ Kit, User Manual

   http://www.olink.com/products/document-download-center/)
   User Manual
   Protein list Inflammation
   Validation data package Immuno-Oncology panel

3. MATERIALS AND EQUIPMENT
   - 3.1. Proseek multiplex probe kit $^{96\times96}$ (store at +4°C)
   - 3.2. Proseek multiplex detection kit $^{96\times96}$ (store at -20°C)
   - 3.3. Proseek multiplex controls (store at -20°C)
   - 3.4. Pipettes and Pipette filter tips
   - 3.5. 96 Well Multiply-PCR Plate half skirt
   - 3.6. Microcentrifuge tubes and Falcon™ 15mL Conical Centrifuge Tubes
   - 3.7. 8-well strips tube with lids
   - 3.8. Multi-channel pipette reservoir
   - 3.9. Adhesive plastic film (heat-resistant)
   - 3.10. High purity water (sterile filtered, MilliQ® or similar)
   - 3.11. Microcentrifuge for tubes and Centrifuge for plates
   - 3.12. Vortex
   - 3.13. VWR Reagent reservior 25ml
   - 3.14. Freezing block (-20°C) for enzyme handling
   - 3.15. Refrigerator or cold room (+2°C to +8°C)
   - 3.16. Bio-RAD T100 Thermal Cycle
   - 3.17. Fluidigm BioMark™ HD System
   - 3.18. Juno System

4. SAFETY
   - 4.1. Wear protective gloves and lab coat while performing this procedure
   - 4.2. Perform all open-vessel work within a laminar flow biological safety cabinet to avoid contamination of samples or operator exposure to potentially infectious materials
   - 4.3. Dispose of all biological waste within the appropriate waste containers

5. PROCEDURE
   - 5.1. Overview of the assay



5.2. Day 1: Sample incubation with Probes

Important Note: All the vortex steps require 30s vortex time, expect of Enzyme and PCR polymerase

5.2.1. Thaw samples, vortex and spin down at 400 × g, 1 min at RT

5.2.2. Thaw the Incubation Stabilizer from the Proseek Multiplex Controls box in RT, vortex 30s and spin briefly, place on ice

5.2.3. Thaw the interplate control (IPC) and negative control from the Proseek multiplex controls box in RT, vortex 30s and spin briefly, place on ice; prepare an 8-strip tube, mark the negative control on the top. Add 3x5 µL Negative Control and 3x5 µL IPC in a 8-strip tube, SEE figure 1

Fig 1 Add NC and IPC in 8-strip tube

5.2.4. Prepare incubation mix in 1.5mL microcentrifuge tube, vortex 30s and spin each reagent before transfer to the mix, Pipette the incubation solution carefully to avoid foaming

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

5.2.5. Vortex the incubation mix for 30s and spin down the content. Transfer 47 µL per well of the Incubation mix to a new 8-well strip. Vortex the strip tube for 10s and spin down.

5.2.6. Prepare a 96-well plate and label as Incubation plate. Mark the control position on the plate according to plate layout (Figure 2).Use a multi-channel pipette to transfer 3µL of the Incubation mix from the 8-well strip to the bottom of each well of a 96-well plate by using reverse pipetting. Pipette from the uppermost part of the Incubation mix to prevent liquid from sticking to the outside of the pipette tip. Do not change pipette tips

5.2.7. Use multi-channel pipette to add 1µL of each sample to the bottom of sample well of the incubation plate, according to the plate layout in Figure 2

5.2.8. Use a multi-channel pipette to add 1µL of negative control and IPC from 8-strip to the bottom of the well in position C12-H12, according to the plate layout in Figure2

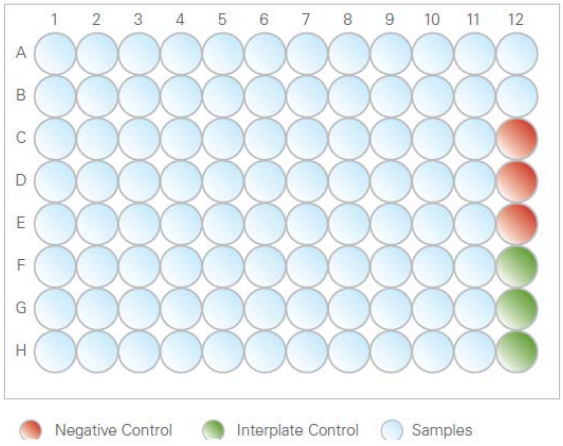


Figure 2. Incubation plate layout

5.2.9. Seal the incubation plate with an adhesive plastic film. It is important that all wells are properly sealed, especially around the edges to avoid evaporation of samples. Vortex the incubation plate for 30s and Spin down the content at 400 × g, 1 min at room temperature

5.2.10. Incubate the Incubation Plate overnight at +2°C to +8°C. **Be consistent for the incubation time between 16-18 hrs**

5.3. Day 2: Extension of the Conjugated Oligonucleotide

5.3.1. Turn on your thermal cycler (HIMC Bio-Rad T100 Thermal Cycle) and create a PEA (Proximity Extension Assay technology) program on the thermal cycler with the following conditions; Preheat the PCR machine by running this protocol and pause when sample temperature reaches to 50C.

Extension	50°C	20 min	
Hot start	95°C	5 min	
PCR Cycle	95°C	30 s	×17
	54°C	1 min	
	60°C	1 min	
Maintain the reaction at	10°C	∞, hold	

5.3.2. Thaw the PEA Solution, vortex 30s and spin briefly. Prepare the following extension mix in a 15ml Falcon tube. Use ice box when removing the PEA Enzyme and the PCR Polymerase from -20°C. Vortex 10s and spin down the content briefly before pipetting the enzymes into the mix. Keep the leftover of the PCR polymerase in the ice box, which will be used for the further step

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

5.3.3. Vortex the Extension mix for 30s
5.3.4. Bring the Incubation Plate to room temperature and spin down at 400 × g, 1 min at RT
5.3.5. Pour the Extension mix into a multi-channel pipette reservoir
5.3.6. Carefully remove the plastic adhesive film from the Incubation Plate
5.3.7. **Steps 5.2.7-5.2.10 have to be performed within 5 minutes**; Start a timer before transfer 96 µL of Extension mix to each well of the Incubation Plate by using reverse pipetting; Do not change pipette tips, place the tips against the upper parts of the well wall
5.3.8. Make sure the tips never come in contact with the contents of the wells
5.3.9. Add a new aluminum seal film to the Incubation Plate; It is important that all wells are properly sealed, especially around the edges to avoid evaporation of samples
5.3.10. Vortex the plate thoroughly for at least 30s and ensure that all wells are mixed. Spin down the content at 400 × g, 1 min at RT
5.3.11. Stop the PCR machine which was paused before, and wait the sample temperature to cool down to 30C. After the 5 min timer done for previous step, place the Incubation Plate in the preheated thermal cycler and run the PEA program. This program will be approximately 1 h 40 min
5.3.12. Continue with the Detection step or store the Incubation Plate for up to one week at +4°C

5.4. Detection of targeted oligos throughout real-time PCR

5.4.1. Turn on the "Juno System" and verify that the HX interface plate has been correctly installed. Otherwise, change the Interface plate according to the following instruction: Tap "Tools" and "interface plate" bottom in the screen and select "Switch interface plate". Place the HX interface plate and the loading fixture on the tray and tap "Install". After the interface plate is installed, remove the loading fixture and tap "Close"

5.4.2. Take a new 96.96 Dynamic Array IFC. Inject control line fluid into each accumulator on the chip. Remove and discard the black protective film from the bottom to the chip. Place the chip into the IFC controller then run the "Primer" script. The Primer program will takes approximately 20 mins. Chip needs to be loaded within 30mins after completed Primer, otherwise chip need to be primer again

5.4.3. Thaw the Primer Plate (offered in the kit) at RT, Vortex the plate for 10s after thawing

5.4.4. Thaw the Detection Solution, vortex 30s and spin briefly; prepare the following Detection mix in a 1.5ml microcentrifuge tube; vortex 10s and spin down Detection Enzyme and PCR Polymerase briefly before pipetting the enzymes into the mix

Detection mix	per 96-well plate (µL)
Detection Solution	550.0
High Purity Water	230.0
Detection Enzyme	7.8
PCR Polymerase	3.1
Total	790.9

5.4.5. Vortex 30s of the Detection mix and spin briefly; transfer 95 µL of the Detection mix per well to an 8-well strip. Vortex the strip tube for 10s and spin down

5.4.6. Label a new 96-well plate as "Sample Plate"; use multi-channel pipette to transfer 7.2 µL of Detection mix to each well of a new 96-well plate by reverse pipetting

5.4.7. Remove the Incubation Plate from the thermal cycler; vortex the plate for 30s and spin down the contents

5.4.8. Carefully remove the plastic film and transfer 2.8 µL from each well of the Incubation Plate to the Sample Plate

5.4.9. Seal the Sample Plate with a new plastic adhesive film, vortex the plate for 30s and spin with Primer Plate at 400 × g, 1 min at RT

Figure 3 Loading of primers and samples to the 96.96 Dynamic Array IFC

5.4.10. Gently remove the Primer Plate sealing to avoid contamination between wells; transfer 5 µL from each well of the Primer Plate to the primed 96.96 Dynamic Array IFC by using reverse pipetting; change pipette tips after each primer; Primers are loaded into their respective inlets on the left side of the chip according to Figure 3

5.4.11. Transfer 5 µL from each well of the Sample Plate into the inlets on the right side of the chip according to Figure 3 by reverse pipetting; change pipette tips after each sample; See Figure 3 for a detailed instruction on sample loading

5.4.12. Remove any visible bubbles, e.g. with syringe needle

5.4.13. After the samples and primers are loaded in all wells, place the chip with barcoding facing you in the IFC Controller for loading. Select “load” and “load mix 96.96 GE IFC” followed by “Run Script” to load the assay and sample mixes into the central portion of the Dynamic array. The Program will takes approximately 92 mins

5.4.14. Press the on/off button (on the right side of the instrument) to turn on the Biomarker and press the round button (on the left side) to connect the computer

5.4.15. Open the “Biomarker Data Collection” and select the “start a new run” on the main menu, the lid will open and the tray come out on the Biomarker

5.4.16. After the “Load mix 96.96 GE IFC” program is done, “Eject” the chip from the “Juno” system and remove the tiny dust from the Central Portion using the cello-tape, do this

even though no visible dust. Take a piece of tape and let the sticky part touch (do not press down) the surface of the chip.

5.4.17. Load the IFC chip into the “Biomarker” tray with the barcode facing outwards and Select “Load”, the chip is now loaded into the instrument.

5.4.18. Select “Next” once the barcode and chip type is read and visible in next page. Select the “This is a new chip run” on this page and name the plate using HIMC format Then press “Next”

5.4.19. Set the following options for application reference and probe setting according to Figure 4, then select “Next”

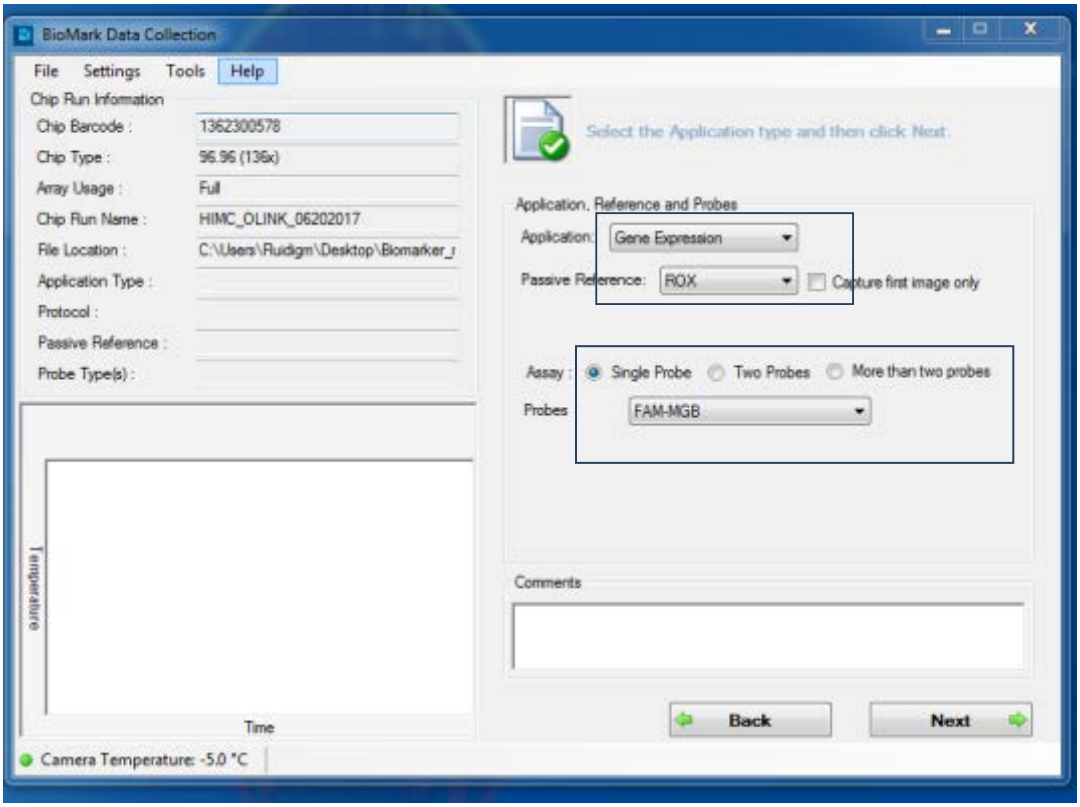


Figure 4 Application reference and probe setting

5.4.20. Select the “Olink Protein Expression 96x96.pcl” protocol from the Olink folder in desktop. Verify the protocol according to Figure 5.Then “Next”

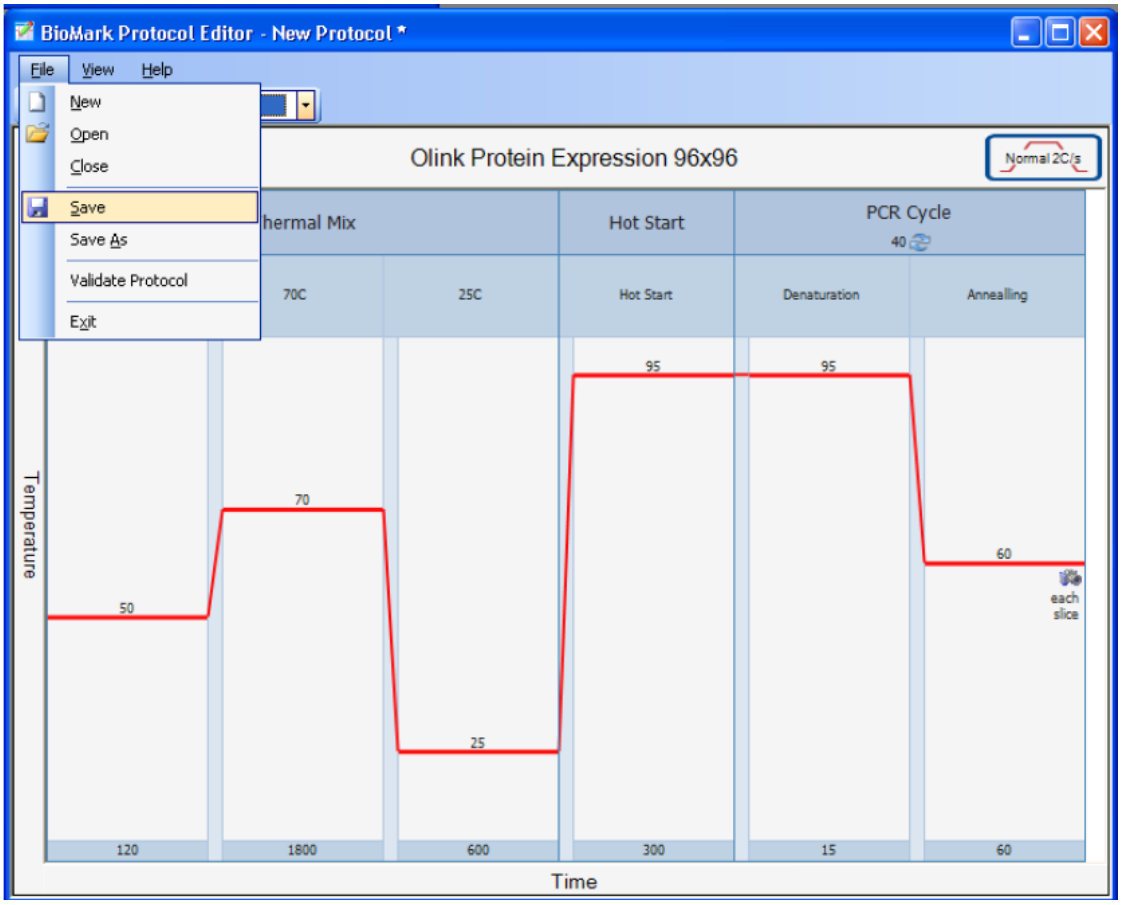


Figure 5 Olink Protein Expression 96x96 Program

5.4.21. Press “Start run” to start the program. <u>The PE Program will take approximately 2 hours.</u> There will be a message on screen “The Run has been successfully completed” after Run is finished.

5.4.22. “Eject” the Chip from the Biomarker instrument.

5.4.23. Use a OLINK assay dedicated Flash Drive, copy the <u>study whole Folder</u> with Run name on Flash drive. Eject the flash drive.

5.4.24. If not running any more Chips on Bio-Mark of the same day, close the Bio-Mark software, shut down the Computer and turn off the Bio-Mark machine power button.

5.4.25. Follow the SOP “HIMC-4031_OlinkPEA_DataAnalysis”.