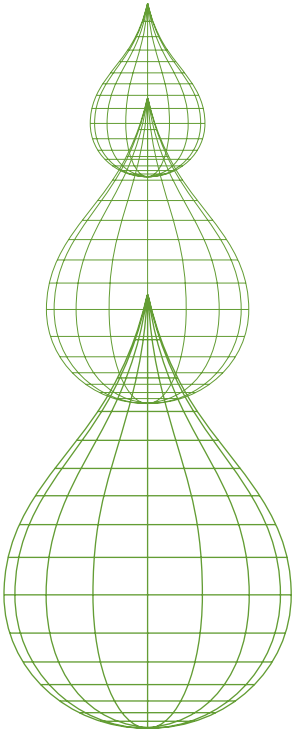
Proseek® Assay Development kit

USER MANUAL



OLINK
BIOSCIENCE

The protocols in this manual are compatible with Proseek Probemaker A (Art. no. 93001-0010), Proseek Probemaker B (Art. no. 93002-0010) and Proseek Assay Reagents in reactions/size 100 and 1000 (Art. no. 93003-0100 and Art. no. 93003-1000).

Table of content

1. Introduction

Proseek® reagents from Olink® Bioscience enable detection and quantification of proteins in a solution, such as plasma and serum, in a minimal sample volume. With the Proseek Assay Development kit a new assay for any target protein with appropriate available antibodies can rapidly be developed. Combining the target specific antibodies with the Proseek reagents, the protein of interest is detected using standard real-time PCR.

The Proseek reagents are based on PEA, which is a Proximity Extension Assay technology. A pair of oligonucleotide labeled antibodies, Proseek probes, are allowed to pair-wise bind to the target protein present in the sample in a homogeneous assay, without washing steps. When the two Proseek probes are in close proximity, a new PCR target sequence is formed by a proximity dependent DNA polymerization event. The resulting sequence is subsequently detected and quantified using real-time PCR.

2. Principle of the assay

1: Create Proseek probes A and B by conjugating your antibodies to Oligo A and B using Proseek Probemaker A and B.

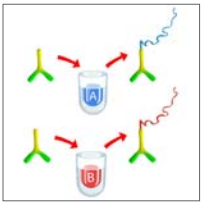


Fig 1.

2: Make a dilution series of your antigen standard in Calibrator Diluent.

Fig 2.

3: Incubate dilution series and samples with Proseek probes A and B.

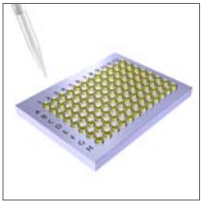


Fig 3.

4: During the incubation, Proseek probes will bind to the target protein.

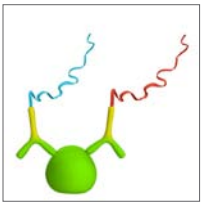


Fig 4.

5: Add the Pre-Extension Solution to dilute the Proseek probes and lower their effective concentration. Subsequently, add the Extension master mix which will extend oligos of two Proseek probes that are bound to a target protein through a DNA polymerization event, creating the real-time PCR amplicon.

Fig 5.

6: Combine the Real-Time PCR master mix with the extension products.

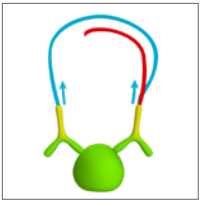


Fig 6.

7: Amplify the DNA using your standard real-time PCR instrument.

Fig 7.

8: Analyze your real-time PCR data in a spreadsheet software, e.g. Microsoft® Excel®.

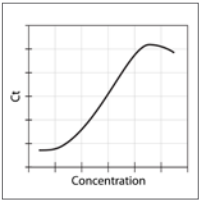


Fig 8.

3. Application

3.1 DETECT AND QUANTIFY PROTEIN EXPRESSION

Proseek is intended to be used for detection and quantification of a single protein in a solution, e.g. plasma and serum. 1 µl of sample is needed per reaction and the target protein is detected using one single antigen affinity purified polyclonal antibody batch or two matched monoclonal antibodies.

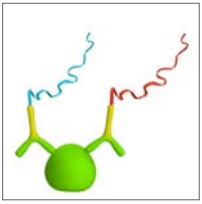


Fig 9. Single protein detection.

4. Reagents and equipment

4.1 PROSEEK ASSAY DEVELOPMENT KIT REAGENTS

When setting up a new Proseek assay you need the following Proseek Assay Development kit components:

- Proseek Probemaker A – to create Proseek probe A
- Proseek Probemaker B – to create Proseek probe B
- Proseek Assay Reagents – to perform the assay

4.1.1 PROSEEK PROBEMAKER

Proseek Probemaker enables conjugation of an antibody to Oligo A or B, creating Proseek probe A or B. One Proseek Probemaker (A or B) can be used for only one antibody conjugation. Proseek probes from one conjugation is sufficient for approximately 100 000 reactions.

Each Proseek Probemaker box contains the following:

- Oligo A or B – *One vial with lyophilized activated oligonucleotide for conjugation of 10 µg antibody at a concentration of 1 mg/ml.*
- Conjugation Buffer (ready-to-use) – *Reagents for buffering the conjugation reaction.*
- Stop Reagent (ready-to-use) – *Reagents for stopping the conjugation reaction.*
- Storage Solution A or B (ready-to-use) – *Reagents for preserving the Proseek probe A or B. The probe is stable in the Storage Solution for 12 months at +4°C.*

4.1.2 PROSEEK ASSAY REAGENTS

Proseek Assay Reagents contains all necessary reagents to perform the Proseek Assay protocol. Proseek Assay Reagents is available in kit sizes of 100 and 1000 reactions.

Each Proseek Assay Reagents box contains the following:

- Probe Diluent (ready-to-use) – *For dilution and 3-month storage of Proseek probes.*
- Calibrator Diluent (ready-to-use) – *For dilution of antigen standard and sample (if necessary).*
- Assay Solution (ready-to-use) – *For combining with Proseek probes to set up incubation with samples and diluted antigen standard.*
- Pre-Extension Solution (10x) – *For dilution of the incubation reaction.*
- Extension Solution (10x) – *Contains all the components needed for the extension of the Proseek probe oligos except for the Extension Polymerase.*
- Extension Polymerase (5 U/µl).
- Real-Time PCR Solution (ready-to-use) – *Contains all the components needed for real-time PCR detection and quantification except for the Real-Time PCR Polymerase.*
- Real-Time PCR Polymerase (1 U/µl).

4.2 REAGENTS TO BE SUPPLIED BY THE USER

- One single antigen affinity purified polyclonal antibody batch or two matched monoclonal antibodies
- Antigen standard, preferably from the same supplier as for the antibody (recommended)
- High purity water (distilled, MilliQ® or similar)

4.3 EQUIPMENT NEEDED

- Microfuge tubes
- 96-well microplate or test tubes (typically PCR tubes)
- Optical 96- or 384-well microplate or test tubes (for real-time PCR detection)
- Pipettes (covering the range from 1 µl to 1000 µl)
- Multi-channel pipettes (recommended)
- Pipette tips
- Freezing block (-20°C) for enzymes
- Thermal cycler with heated lid
- Incubator or oven, +37°C
- Vortexer
- Centrifuge for plates or tubes
- Adhesive plastic film
- Optical adhesive plastic film (for real-time PCR detection)
- Real-time PCR instrument; Olink has tested Applied Biosystems 7900, Applied Biosystems StepOne and Stratagene MX3000P

4.4 SOFTWARE FOR ANALYSIS

The result from a Proseek experiment is a result file with Ct values obtained from the real-time PCR instrument. This result file can easily be exported to a spreadsheet program, e.g. Microsoft Excel, where average Ct, ΔCt and standard deviations can be calculated and further converted to concentrations.

5. Assay considerations

5.1 ANTIBODIES

Your choice of antibody or antibodies is crucial when setting up the Proseek assay. For conjugation with Proseek Probemaker you can use either one single polyclonal batch or two matched monoclonals. The antibody or antibodies should meet the following criteria:

- If a polyclonal antibody is used, it must be raised against the whole native protein or at least large fragments (not peptides) and be antigen affinity purified (not IgG fraction).
- The antibody must have a concentration of 1 mg/ml (10 µl is needed per conjugation).
- The antibody has to be in an amine free buffer, ideally PBS. Low levels of BSA (0.1%), trehalose (5%) and sodium azide (0.02%) are tolerated.

If you are unsure about the composition of the buffer your antibodies are stored in, Olink strongly recommends dialysis or buffer exchange prior to conjugation. Antibodies from various sources and suppliers vary in residual primary amine content often in the form of remaining glycine from the acidic elution of the antibody during the antigen-affinity purification step. Even if the supplier states PBS content, it is likely that there are residual amounts of primary amines. Antibodies from R&D Systems are suitable to conjugate directly with Proseek Probemaker and do not require dialysis.

Olink recommends the following standard procedure for buffer exchange:

- Pre-equilibrate an illustra MicroSpin G-50 Columns spin column (GE Healthcare, Art. no. 27-5330-01) with 1xPBS by first spinning the column at 3000 rpm for 1 min, then add 400 µl of 1xPBS and spin again for 1 min and repeat 4 times. Place the column in a new microfuge tube.
- Add your antibody (25–50 µl) to the column and spin again for 2 min at 3000 rpm. The concentration of the collected antibody should be verified by OD. 1 mg/ml should have an OD 280 nm of 1.4.

Concentrating low concentration antibodies prior to Proseek Probemaker conjugation is not recommended unless you have large milligram amounts since losses are very high with filter type concentrations.

5.2 SAMPLE MATERIAL

Proseek has so far shown to be successful with the following sample types:

- Serum
- EDTA plasma
- Citrate plasma
- Cerebrospinal fluid (CSF)

Note: *Do not use heparin treated plasma since it will inhibit the real-time PCR reaction.*

5.3 VALIDATION OF ASSAY

To be able to properly evaluate your results it is advisable to do a simple validation of your assay, e.g. by assessing sensitivity, dynamic range, precision, recovery and linearity of dilution.

5.3.1 BACKGROUND

By adding the Calibrator Diluent only (without the antigen) you will obtain a value for the background level of your assay.

5.3.2 CALIBRATION CURVE: SENSITIVITY, DYNAMIC RANGE AND PRECISION

The calibration curve, made from a dilution series of the antigen standard in replicates will give information about the sensitivity, dynamic range and precision of your assay. Sensitivity, or limit of detection, is defined as the lowest detectable concentration that significantly exceeds the background level and is calculated as the obtained background concentration +2 standard deviations. The precision can be improved by increasing the number of replicates.

5.3.3 RECOVERY

You will get an indication of the recovery by spiking in the antigen standard at a certain concentration within the dynamic range of the calibration curve into one of your samples. Recovery is defined as the ratio of the measured concentration of the spiked-in protein in the sample (after subtracting the endogenous concentration) and the measured amount of the spiked-in protein in the Calibrator Diluent, expressed as a percentage.

5.3.4 LINEARITY OF DILUTION

Sample matrix effects can be investigated by spiking in the antigen standard at a high concentration into one of your samples followed by a serial dilution of the spiked-in sample. The resulting dilution curve should be linear if no matrix effects are present in the sample.

6. Reagent handling and storage

6.1 PROSEEK PROBEMAKER

Store the unused Proseek Probemaker kit at -20°C. Once you have conjugated your antibody with Proseek Probemaker we recommend storage at +4°C in Storage Solution A or B. The Storage Solution A and B contains buffer and reagents for stabilizing and preserving Proseek probes A and B.

Each Proseek Probemaker kit (A or B) contains reagents to conjugate 10 µg antibody at a concentration of 1 mg/ml. Proseek probes from one conjugation is sufficient for approximately 100 000 reactions.

Oligo A or B – store at -20°C

- One vial with lyophilized activated oligonucleotide for one conjugation of 10 µg antibody at a concentration of 1 mg/ml.

Conjugation Buffer – store at -20°C

- Thaw, vortex and quick spin before use
- Ready-to-use solution

Stop Reagent – store at -20°C

- Thaw, vortex and quick spin before use
- Ready-to-use solution

Storage Solution A or B – store at -20°C

- Thaw, vortex and quick spin before use
- Ready-to-use solution

6.2 PROSEEK ASSAY REAGENTS

Store the unused Proseek Assay Reagents at -20°C. Some solutions are supplied as 10x concentrated stocks. Dilute required volumes of the stocks immediately before use.

Note: *Do not store diluted reagents.*

Calibrator Diluent – store at -20°C

- Thaw, vortex and quick spin before use
- Ready-to-use solution

Probe Diluent – store at -20°C

- Thaw, vortex and quick spin before use
- Ready-to-use solution

Assay Solution – store at -20°C

- Thaw, vortex and quick spin before use
- Ready-to-use solution

Pre-Extension Solution (10x) – store at -20°C

- Thaw, vortex and quick spin before use
- Dilute 1:10 in high purity water immediately before use

Extension Solution (10x) – store at -20°C

- Thaw, vortex and quick spin before use
- Dilute 1:10 in high purity water immediately before use

Extension Polymerase (5 U/µl) – store at -20°C

- The Extension Polymerase should be kept at -20°C at all times. Use a freezing block (-20°C) when removing the enzyme from the freezer.
- Quick spin the Extension Polymerase before adding to the Extension Solution and high purity water at a 1:100 dilution immediately before use.
- Ensure that the Extension master mix is thoroughly vortexed before addition to the samples.

Real-Time PCR Solution – store at -20°C

- Thaw, vortex and quick spin before use
- Ready-to-use solution

Real-Time PCR Polymerase (1 U/µl) – store at -20°C

- The Real-Time PCR Polymerase should be kept at -20°C at all times. Use a freezing block (-20°C) when removing the enzyme from the freezer.
- Quick spin the Real-Time PCR Polymerase before adding to the Real-Time PCR Solution at a 1:30 dilution immediately before use.
- Ensure that the Real-Time PCR master mix is thoroughly vortexed before addition to the samples.

7. Protocols

7.1 PROSEEK PROBEMAKER PROTOCOL

The following Proseek Probemaker protocol describes how you conjugate either the Oligo A or the Oligo B to one antibody, to create a Proseek probe A or B. You can create the Proseek probes A and B in parallel, however it is highly important to change pipette tips and gloves between the A and B conjugation in order to avoid cross-contamination between the probes. If a single polyclonal antibody batch is used, split the antibody into two separate tubes with 10 µl in each and label one with Oligo A and the other with Oligo B.

Before starting:

1. Please read through the entire Proseek Probemaker protocol.
2. Ensure that your antibody or antibodies to be conjugated meet the requirements specified in Section 5.1.

	CONJUGATION OF PROSEEK PROBEMAKER OLIGO A OR B TO ANTIBODY
1)	Add 1 µl of Conjugation Buffer to 10 µl of the antibody to be conjugated.
2)	Mix gently with a pipette.
3)	Transfer the antibody solution to one vial of lyophilized Oligo (A or B).
4)	Mix gently with a pipette and make sure the oligo is completely dissolved.
5)	Incubate for 3 h at +37°C.
6)	Add 1 µl of Stop Reagent to the reaction.
7)	Agitate gently.
8)	Incubate for 30 min at room temperature.
9)	Transfer 9.6 µl of the reaction to the vial with Storage Solution (A or B). *Store the few µl that are left of the reaction in the Oligo vial at +4°C if you would like to verify the conjugate with gel electrophoresis.*
10)	Incubate for 30 min at room temperature.
11)	The conjugation is now completed and your Proseek probe is stable in the Storage Solution for 12 months at +4°C. Write the name of the antibody that was conjugated on the new label (Probe A or Probe B, 130 nM) and put it over the old label. Note: *Stability may vary between antibodies.*

7.2 PROSEEK ASSAY PROTOCOL

The following Assay protocol describes how you perform the Proseek assay in a 96-well microplate but you can as well use test tubes (PCR type). For the real-time PCR detection you will need either an optical 96- or 384-well plate or optical test tubes. It is advisable to use a multi-channel pipette in the reagent transfer steps when it is possible. For loading the multi-channel pipette you can transfer the master mix to an 8- or 12-tube strip. Change pipette tips between sample and reagent transfer steps to avoid cross-contamination.

There is always a loss of volume when pipetting and transferring reagents; therefore always prepare master mixes for additional reactions. This has also been taken into account in the Proseek Assay Reagents where Assay Reagents in kit size of 100 reactions contains reagents for approximately 120 reactions and Assay Reagents in kit size of 1000 reactions contains reagents for approximately 1200 reactions. If your total number of reactions in one experiment is 40 or more, we recommend you to prepare master mixes for 8 additional reactions.

Before starting:

1. Please read through the entire Proseek Assay protocol.
2. Decide how many samples you will include in the experiment and the number of replicates.
3. Plan how to prepare the calibration curve; number of concentrations, dilution factor and number of replicates.
4. Calculate the total number of reactions in the experiment.
5. Use the 96-well plate template in Appendix A on page 24 and select a location for each reaction.

1.	DILUTION OF PROSEEK PROBES FOR 3-MONTH STORAGE
a)	Thaw the Probe Diluent, vortex and quick spin.
b)	Dilute Proseek probes A and B (from Probemaker Storage Solution, 130 nM) 1:200 in the Probe Diluent in separate tubes: add 1 µl of each Proseek probe to 199 µl of Probe Diluent. *The final concentration is now 650 pM, the Proseek probes can be stored in the Probe Diluent for 3 months at +4°C.*

2.	PREPARATION OF CALIBRATION CURVE AND SAMPLES
a)	Thaw the Calibrator Diluent and the Assay Solution, vortex and quick spin.
b)	Prepare a serial dilution of your antigen standard in the Calibrator Diluent. *We recommend 7 concentrations in dilution steps of 1:10 in the calibration curve, e.g. 10 nM, 1 nM, 100 pM, 10 pM, 1 pM, 0.1 pM and 0.01 pM, and a zero standard (0 pM). 1 pM of a 10 kDa antigen corresponds to 10 pg/ml). After each dilution, mix gently by pipetting and then change to a new pipette tip.*
c)	If necessary, dilute your samples in the Calibrator Diluent.

3. PROBE INCUBATION

a) Prepare a probe master mix by mixing Proseek probes A and B from step 1b) with the Assay Solution according to the table below and vortex briefly. *3 µl of probe master mix is needed per reaction; prepare an excess volume to account for pipetting losses.*

	1 reaction (µl)	..... reactions (µl)
Proseek probe A (650 pM)	0.3	.....
Proseek probe B (650 pM)	0.3	.....
Assay Solution	2.4	.....
Total volume of probe master mix	**3**	.....

b) Transfer 3 µl of the probe master mix to each well of a 96-well plate (or multi-tube strip). *It is advisable to transfer the probe master mix to an 8- or 12-tube strip and use a 1–10 µl multi-channel pipette when subsequently transferring to the plate.*

c) Add 1 µl of sample or diluted antigen standard to the 96-well plate.
Note: *Do not mix by pipetting up and down since bubbles will form, instead centrifuge the plate briefly after all samples and diluted antigen standards have been transferred to the plate.*

d) Seal the plate with an adhesive plastic film.

e) Incubate the plate for 2 h at room temperature. *This incubation can also be done over night at +4°C.*

4. PRE-EXTENSION AND EXTENSION

a) Thaw the Pre-Extension Solution and the Extension Solution, vortex and quick spin. Pre-heat the thermal cycler to +37°C. Make sure you have the following Extension program ready in the thermal cycler:

- Polymerization: 20 min, +37°C
- Inactivation of enzyme: 10 min, +85°C
- Cooling: ∞, +4°C

b) Prepare a Pre-Extension master mix by mixing the Pre-Extension Solution (10x) with high purity water according to the table below and vortex briefly. *76 µl of Pre-Extension master mix is needed per reaction; prepare an excess volume to account for pipetting losses.*

	1 reaction (µl)	..... reactions (µl)
Pre-Extension Solution (10x)	7.6	.....
High purity water	68.4	.....
Total volume of Pre-Extension master mix	**76**	.....

c) Prepare an Extension master mix by mixing the Extension Solution (10x) and the Extension Polymerase (5 U/µl) with high purity water according to the table below and vortex briefly. Use a freezing block when removing the Extension Polymerase from -20°C. *20 µl of Extension master mix is needed per reaction; prepare an excess volume to account for pipetting losses.*

	1 reaction (µl)	..... reactions (µl)
Extension Solution (10x)	2	.....
Extension Polymerase (5 U/µl)	0.2	.....
High purity water	17.8	.....
Total volume of Extension master mix	**20**	.....

d) Place the plate from 3e) in the pre-heated thermal cycler. Add 76 µl of the Pre-Extension master mix to each well and incubate for 5 min at +37°C without sealing the plate.

e) Transfer 20 µl of the Extension master mix to each well of the plate. Seal the plate with an adhesive plastic film. *The total volume in each well is now 100 µl.*

f) Start the thermal cycler Extension program and use a heated lid:

- Polymerization (100 µl): 20 min, +37°C
- Inactivation of enzyme (100 µl): 10 min, +85°C
- Cooling: ∞, +4°C

If not being able to run real-time PCR immediately after the Extension, the plate can be stored at +4°C for 24 h.

5. REAL-TIME PCR

a) Thaw the Real-Time PCR Solution, vortex and quick spin.
Note: *The real-time PCR instrument should be turned on at least 30 min before starting the amplification.*

b) Prepare a Real-Time PCR master mix by mixing the Real-Time PCR Polymerase (1 U/µl) with the Real-Time PCR Solution according to the table below and vortex briefly. Use a freezing block when removing the Real-Time PCR Polymerase from -20°C. *6 µl of Real-Time PCR master mix is needed per reaction; prepare an excess volume to account for pipetting losses.*

	1 reaction (µl)	..... reactions (µl)
Real-Time PCR Solution	5.8	.....
Real-Time PCR Polymerase (1 U/µl)	0.2	.....
Total volume of Real-Time PCR master mix	**6**	.....

c) Transfer 6 µl of the Real-Time PCR master mix to each well of a new optical 96- or 384-well plate.

d) Add 4 µl of each extension product from step 4f) to the plate.

e) Seal the plate with an optical adhesive plastic film and centrifuge briefly.

f) Place the plate in your real-time PCR instrument.

g) Thermal-cycling conditions:
FAM as reporter and ROX as passive reference (Dabcyl is the quencher, you may denote "none" or "non-fluorescent" with equal performance).

- +95°C, 5 min
- +95°C, 15 s
- +60°C, 1 min

} 45 cycles

Start the run.

When the run is finished, remove and dispose of the plate.

6. ANALYSIS

Export the result file from the real-time PCR instrument to a spreadsheet program e.g. Microsoft Excel. Calculate the average Ct value and standard deviation of replicates for samples and data points in the calibration curve. Plot the calibration curve with the concentrations on the x-axis and the average Ct values (in reverse order) on the y-axis.

8. Results

8.1 TYPICAL RESULTS

```
SDS 2.3 Absolute QuantificationResults  1.0
Filename          PEA 076_110201
PlateID
Assay Type        Absolute Quantification
Run DateTime      2/1/11 2:30:58 PM
Operator
ThermalCycleParams

Sample Information

Position        Flag    Sample  Detector        Task
A1      Passed  A1      FAM     Unknown 34.11196
A2      Passed  A2      FAM     Unknown 34.106636
A3      Passed  A3      FAM     Unknown 33.55748
A4      Passed  A4      FAM     Unknown 33.853817
A5      Passed  A5      FAM     Unknown 33.866272
A6      Passed  A6      FAM     Unknown 33.74429
A7      Passed  A7      FAM     Unknown 33.94533
A8      Passed  A8      FAM     Unknown 34.02659
A9      Passed  A9      FAM     Unknown 33.60669
A10     Passed  A10     FAM     Unknown 33.83272
A11     Passed  A11     FAM     Unknown 33.811447
A12     Passed  A12     FAM     Unknown 33.326935
```

Fig 10. Typical result file from the real-time PCR instrument (Applied Biosystems 7900).

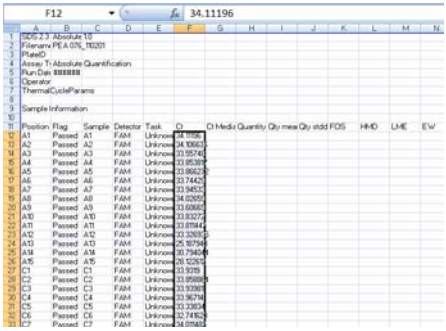


Fig 11. Result file exported to Microsoft Excel.

	Ct Rep. 1	Ct Rep. 2	Avg. Ct	Stdev. Ct
0	33.9	33.7	33.8	0.09
0.01	33.3	32.7	33.0	0.42
0.1	32.0	32.0	32.0	0.00
1	30.9	30.6	30.7	0.21
10	29.0	29.1	29.0	0.04
100	27.1	26.7	26.9	0.26
1000	25.7	25.1	25.4	0.44
10000	27.2	26.6	26.9	0.41

Fig 12. Ct values in duplicates (Rep. 1 and. Rep. 2), average Ct (Avg. Ct) and standard deviation (Stdev. Ct) of each data point in the calibration curve.

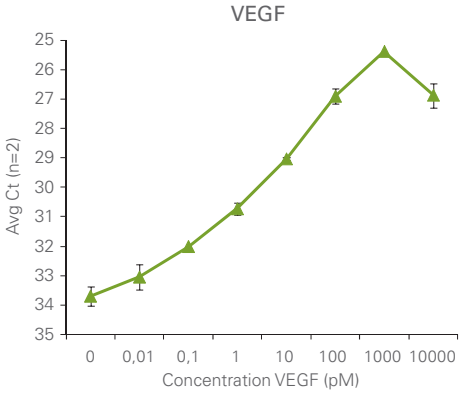


Fig 13. Calibration curve of VEGF (R&D Systems Ab:AF-293-NA and Ag:293-VE-010), 0–10 000 pM, with concentrations on the x-axis and the average Ct values (in reverse order) of duplicates on the y-axis. Error bars indicate ± standard deviation.

8.2 ANALYSIS OF REAL-TIME PCR DATA

8.2.1 ABSOLUTE QUANTIFICATION WITH LINEAR REGRESSION

Calculate the concentrations in the calibration curve to $\log_{10}$ scale in a spreadsheet program e.g. Microsoft Excel; exclude the zero buffer and concentrations that are not within the linear range of the calibration curve. Plot the values in a dot plot graph with the $\log_{10}$ scale concentrations on the x-axis and the average Ct values in reverse order on the y-axis. Use linear regression and display the trend line, the equation (y=kx+m) and the R^2 value in the graph. Use the formula $10^{((m-Ct)/k)}$ to back-calculate the concentration of each Ct value in the calibration curve and your samples.

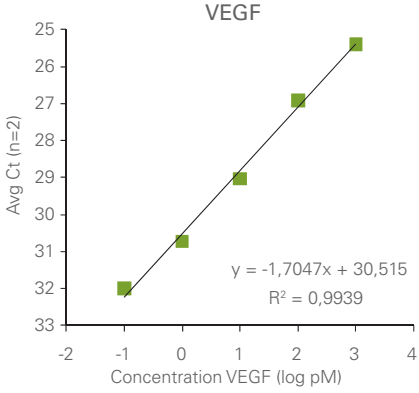


Fig 14. Calibration curve of VEGF, 0.1–1000 pM, with concentrations converted to log10 scale on the x-axis and the average Ct value (in reverse order) of duplicates on the y-axis. Standard linear regression is used.

9. Tips and tricks

9.1 PRECISION

The use of appropriate equipment and pipetting technique are crucial in order to obtain high precision and low standard deviation between replicates; using well calibrated pipettes for small volumes, fine and well attached pipette tips and a consistent and accurate pipetting technique are of utmost importance. Increasing the number of replicates will also improve the precision.

9.2 SENSITIVITY

The sensitivity of your assay may be increased by diluting the Proseek probes, stored in the Probe Diluent at 650 pM, slightly further, e.g. another 1:2 dilution in Probe Diluent before preparing the probe master mix. However, this will give you a background level at a higher Ct value, thus increasing the risk for lower precision. Performing the probe incubation over night at +4°C, instead of 2 h at room temperature, may also help increasing the sensitivity.

9.3 QUANTIFICATION

To enhance the precision further of the quantification you can narrow the concentration range of the calibration curve closer to the protein levels of your samples, and use a smaller dilution factor of the antigen standard, e.g. 1:2 or 1:3.

10. Troubleshooting

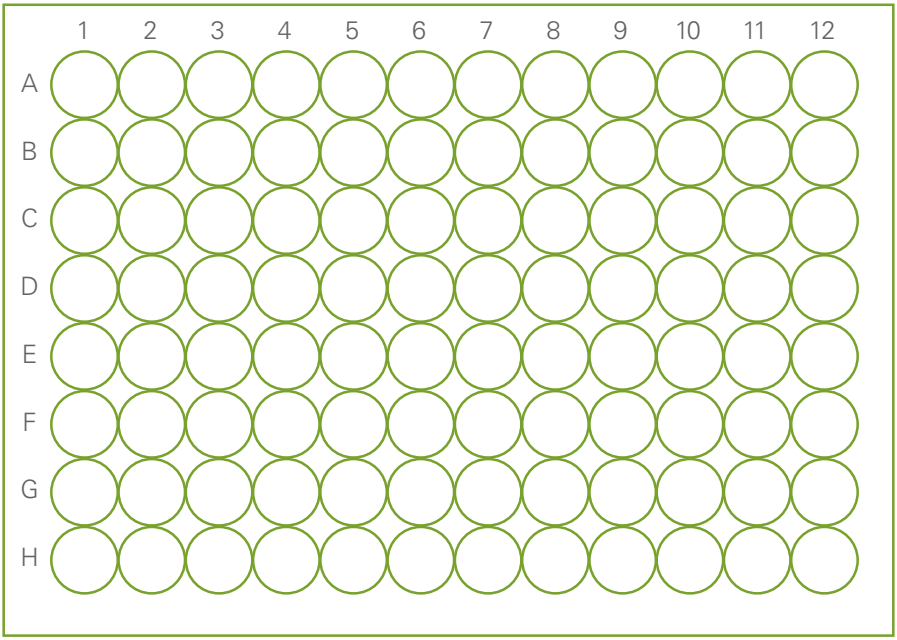
Some general guidelines are given below.

PROBLEM	PROBABLE CAUSE	SUGGESTED SOLUTION
Poor standard curve	No or inefficient conjugation with Proseek Probemaker	Run a polyacrylamide or agarose gel elecrophoresis with your Proseek probes and free antibody to verify a conjugation with Proseek Probemaker. Contact support@olink.com to receive protocols and gel images of expected results.
	No or insufficient binding of Proseek probes to antigen	Check the quality of the antigen. Also ensure that your antibody used for Probemaker conjugation has been raised against the selected antigen; preferably the antigen should be purchased from the same supplier as the antibody.
	Improper dilution of antigen standard	Ensure that the dilution series of the antigen standard has been properly prepared.
	No or inefficient extension reaction	Ensure that the Extension Polymerase has been stored at -20°C.
	No or inefficient real-time PCR amplification	Ensure that the Real-Time PCR Polymerase has been stored at -20°C.
	Wrong settings on the real-time PCR instrument	Ensure that correct settings have been used on the real-time PCR instrument.
	Real-time PCR instrument is not operating well	Run real-time PCR replicates of any real-time PCR master mix (not necessarily Proseek reagents). CV% should be below 8% on a linear scale, if not, recalibrate the instrument and contact the manufacturer.
Low signals in samples	Protein levels in samples are lower than the detection limit	By spiking in the antigen standard into one of your samples at a concentration within the linear range of the calibration curve, you can confirm that a signal over background is detected and the lack of signal in the non-spiked samples is not caused by any sample matrix effects.
	Protein in sample is highly abundant and not within the dynamic range of the calibration curve	Dilute your samples further.

PROBLEM	PROBABLE CAUSE	SUGGESTED SOLUTION
High standard deviation of replicates	Incomplete mixing of reagents	Ensure that all reagents are thoroughly mixed before applying to the reaction plate.
	Uncalibrated pipettes	Ensure that your pipettes are calibrated and that the pipette tips are well attached to the pipettes.
	Uneven pipetting of reagents	Ensure that you use a consistent and accurate pipetting technique during reagent and sample transfer steps. Avoid bubble formation.
	Partial evaporation of samples	Ensure that the adhesive plastic film (or tube lids) is completely sealed during the Probe incubation, Extension and Real-Time PCR.
	Contamination from other wells	Be careful when removing the adhesive plastic film (or tube lids) after the Probe incubation and the Extension to avoid contamination drops from other wells. Before removing the film (or lids) centrifuge the plate briefly. Also remember to change pipette tips between sample and reagent transfer steps.
	Real-time PCR instrument is not operating well	Run real-time PCR replicates of any real-time PCR master mix (not necessarily Proseek reagents). CV% should be below 8% on a linear scale, if not, recalibrate the instrument and contact the manufacturer.

If problems remain, please contact us at support@olink.com or +46 18 444 3970.

Appendix 1 –
96-well plate template



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