



Great design, great data

Olink Webinar Notes

To provide you:

1. Summary of key messages and infographic from Olink bitesize webinar series
2. Further reference materials

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1536, v1.0, 2024-09-13

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I. Proximity Extension Assay (PEA) technology

Learning objectives

- ☐ Understand Olink PEA technology
- ☐ Learn about the technical advantages of PEA
- ☐ Built-in quality control

What is PEA and how it works

The Proximity Extension Assay (PEA) technology combines the best of antibody- and DNA-based methodologies, providing ideal tools for protein biomarker discovery and taking affinity-based protein measurements to a new level. PEA was commercialized by Olink Proteomics AB to develop Olink® biomarker panels. PEA successfully merges a dual-recognition immunoassay with readout from quantitative real-time PCR (qPCR) or Next Generation Sequencing (NGS). This gives a scalable, multiplexed, and highly specific method where thousands of protein biomarkers can be quantified simultaneously. Figures 1-4 illustrate the mechanism and key advantages of PEA.

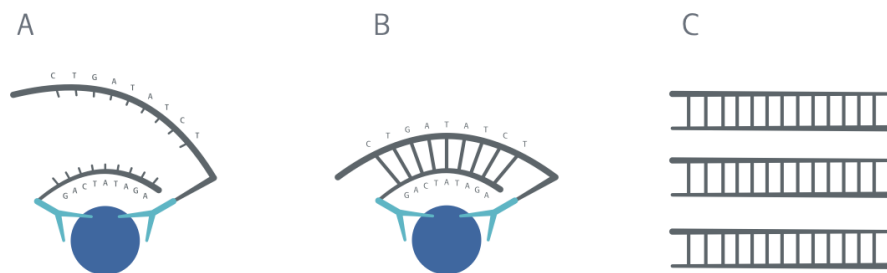


Figure 1. Main pre-readout steps in PEA

- (A) Antibody pairs, labelled with DNA oligonucleotides, bind target antigen in solution.
 (B) Oligonucleotides that are brought into proximity hybridize and are extended by a DNA polymerase.
 (C) This newly created piece of DNA barcode is amplified by PCR and ready for readout by qPCR or NGS.

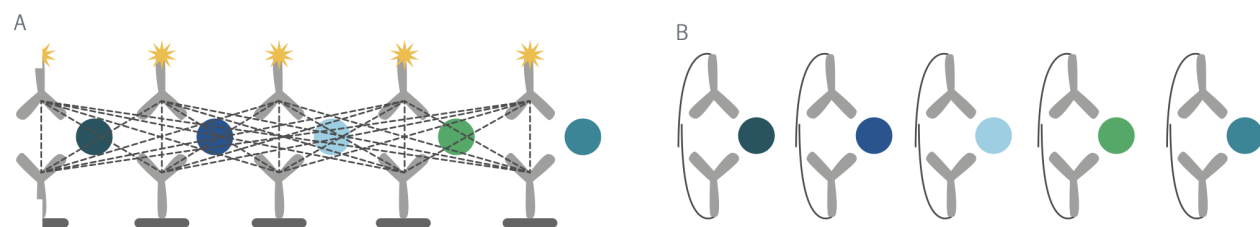


Figure 2. Overcoming the limitations of multiplexed immunoassays

- (A) The problem of cross-reactive interactions when multiplexing a traditional immunoassay.
 (B) Cross-reactive interactions in PEA are not detected.



Figure 3. Barcoding of each protein and sample for NGS readout

The molecular design of the amplicon incorporates a forward and a reverse DNA barcode that contains assay ID information. A third DNA barcode holds the sample ID information. This makes it possible to detect up to 5416 proteins simultaneously while consuming only 2 µL of plasma or serum.

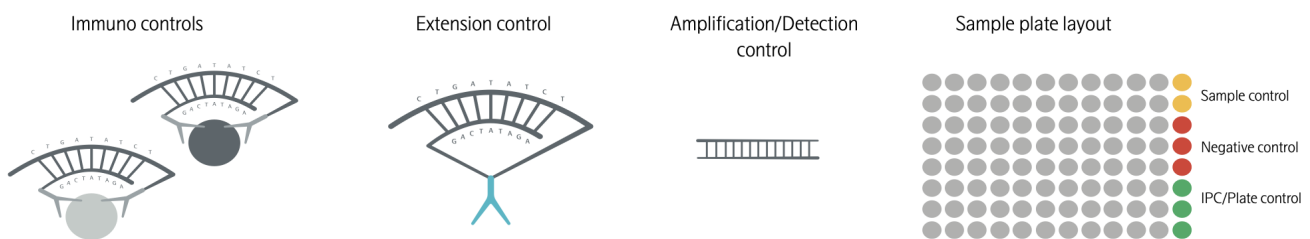


Figure 4. Quality control in PEA

The internal controls monitor each step in the PEA protocol to ensure data quality. Multiplicate of external sample control, negative control and IPC/plate control are included in each sample plate. Sample controls are included in the control strip to estimate the precision. Negative controls serve to measure background levels for each protein and sample plate. IPC/plate controls are used for data normalization for each assay.

How PEA solves historical challenges in proteomics

As illustrated above, PEA solves the historical challenges in proteomics from three aspects (Figure 5 and 6):

1. Dual antibody recognition enhances specificity
2. PCR amplification enables robust sensitivity
3. PEA design and workflow enables low sample input and high multiplexing

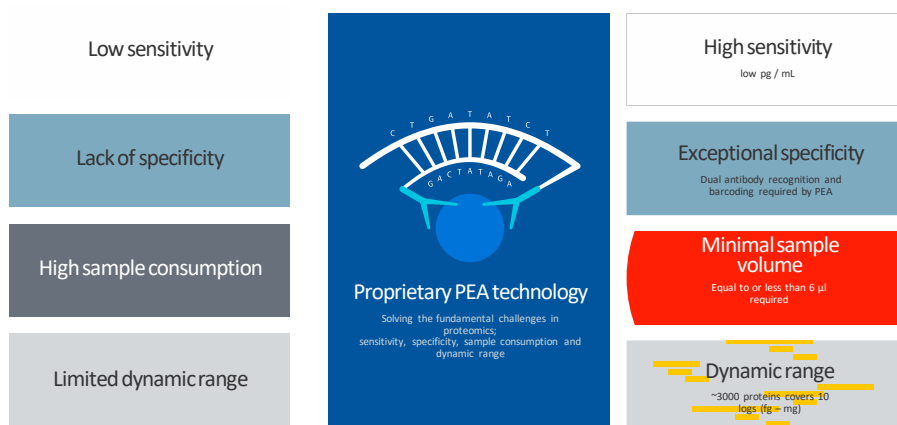


Figure 5. How Olink PEA solves historical challenges in proteomics

Covering a broad range of the plasma proteome

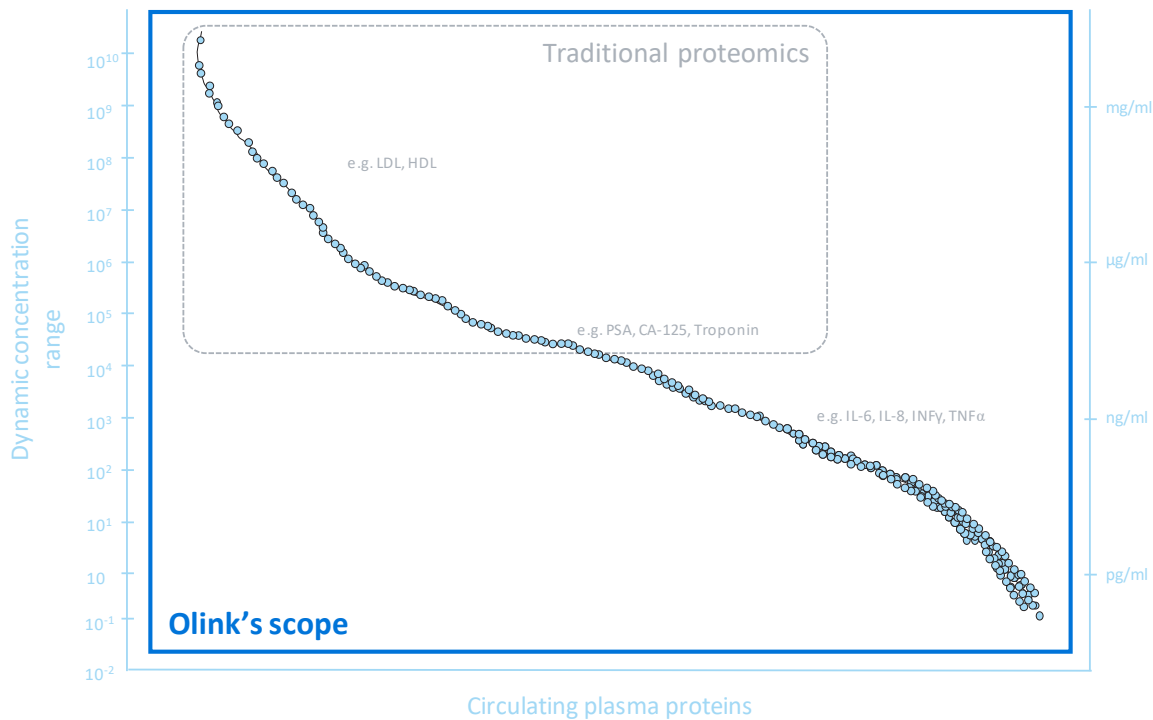


Figure 6. The dynamic range of PEA

Further reading

1. Watch PEA in action– Olink PEA animation 2023
2. Olink Explore – the promise of Proteomics
3. Developed for specificity, validated for quality
4. Olink® White Paper: PEA – a high-multiplex immunoassay technology with qPCR or NGS readout
5. Olink® White Paper - PEA: Exceptional specificity in a high multiplex format

II. Know your question: increase the study power to get actionable data

Learning objectives

- ☐ Understand how the research question defines the study planning
- ☐ Learn how to increase the power of a study

Proteomic research questions

Some examples of applications of Proteomics are shown in Figure 7.

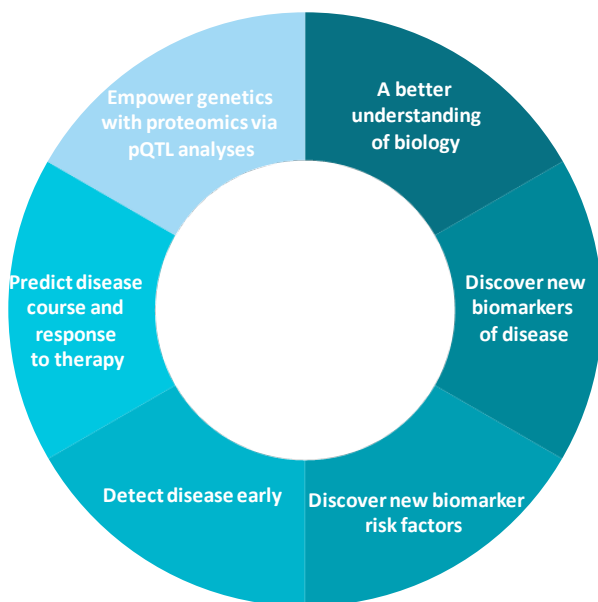


Figure 7. The examples of applications of proteomics

Proteomic research workflow overview and study design scenarios

A typical proteomic research workflow and study design scenarios are illustrated in Figure 8 and 9.

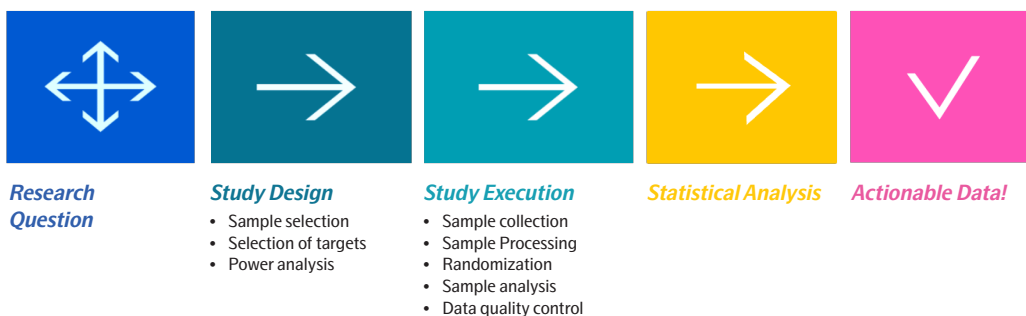


Figure 8. From research questions to actionable data: the overview of proteomics research workflow

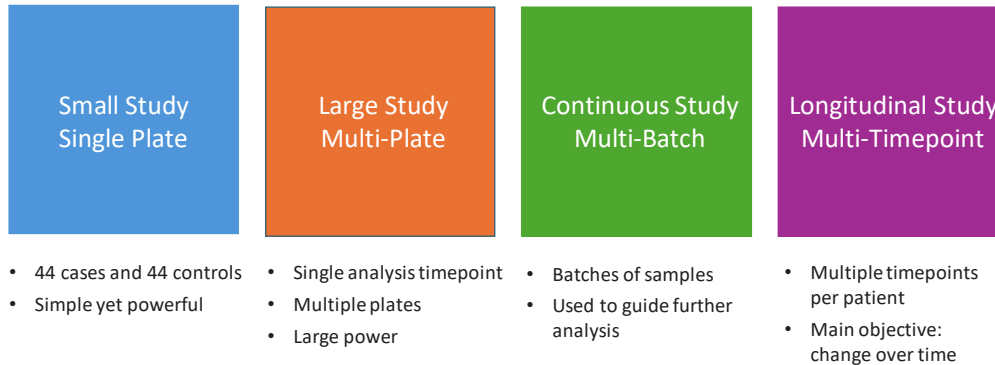


Figure 9. Various proteomics study design scenarios

Study design goals:

1. Maximize power by increasing the number of samples included in the study
2. Minimize experimental variables across the study

The power of a study

The power of a study is defined as the probability that a difference between study groups will be correctly detected, given that the effect is present.

The power of a study depends on the effect size, which is the magnitude of difference between study groups. Effect size is a standardized measurement of the size of the difference we want to detect, and in turn, depends on the size of that difference and the spread or standard deviation of the data.

In the scenario where there is no overlap between the data in the experimental group and the control group, considerable difference is expected. Thereby, the effect size and the study power are high, requiring a smaller sample size. However, if the overlap is larger than the difference between the experimental and control groups, the effect size is less significant, resulting in lower power and a need for a larger sample size.

In line with that, studies are often designed to have 80% power based on assumptions of parameters such as effect size, meaning that there is an 80% chance that we detect a certain difference between two groups. The relationship between effect size and power of the study design is illustrated in Figure 10 and Figure 11.

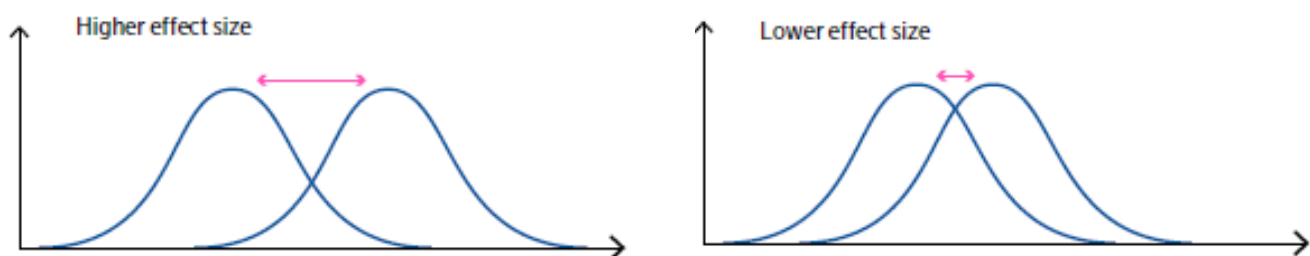


Figure 10. The effect size and power of the study

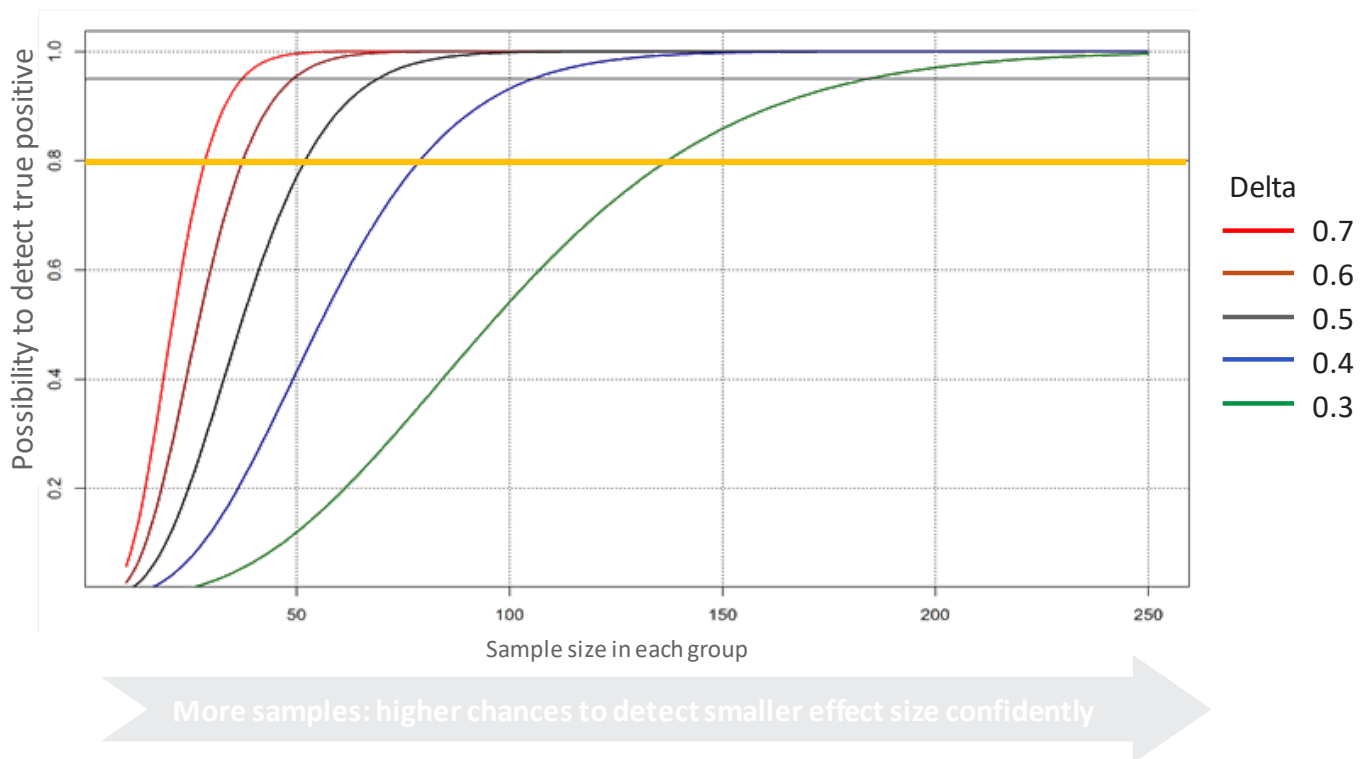


Figure 11. Power calculation example with varying deltas between control and experiment groups

Easy facts to remember about study power

1. If the group size is not the same, power is best increased by enlarging the smallest group.
2. Paired design increases power significantly by reducing biological variations.
3. Effect size has the largest impact on power.
4. Randomization or overlapping/bridging samples are essential for larger studies and for combining studies run at different time points.
5. Always refer to our data science team for help in understanding a study's power ().
6. We offer a free power calculation tool (<https://insight.olink.com/tools/study-size-calculator>).

III. Pre-analytical variation: minimize the non-biological variation in the samples

Learning objectives

- ☐ Understand proper sample selection criteria
- ☐ Learn about non-biological and pre-analytical variation

Sample selection considerations

Sample groups

- Define controls and cases.
- Control group samples are often healthy individuals, but this may not be the best choice depending on the study question.
- Ethnicity, age, sex differences can introduce bias.
- Examples of experimental groups are drug treatment and pathophysiological parameters.

Sample matrix

- Cases and controls must be the same sample matrix.
- Different blood collection methods are considered different matrices.
- Not recommended to compare data across different matrices.

Compatible sample types

The common compatible sample types for PEA are shown in Figure 12.

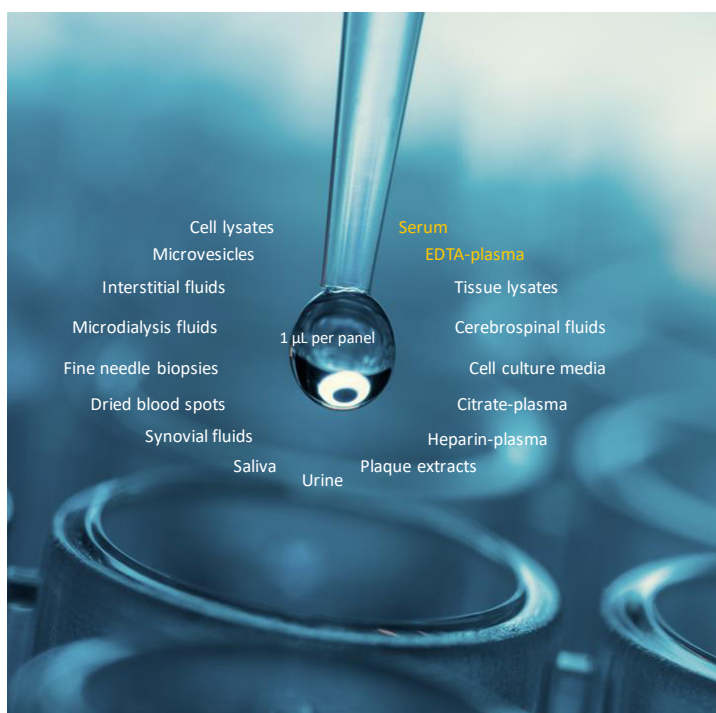


Figure 12. Compatible sample types for PEA

Pre-analytical factors

Temperature and duration of sample handling

- Effect of centrifugation delay on protein stability is minimized when samples are handled at 4°C.
- The pre-centrifugation delay time should be kept short and as consistent as possible between samples.

Temperature is a major variable in protein handling. Upon sample collection, temperature fluctuations from handling to transportation to long-term storage impact the quality of samples. Protein stability and enzyme activity are temperature dependent. Protein degradation and cell lysis can be reduced by keeping samples cold during handling and transportation. Samples should be transported on dry ice to minimize the degradation of proteins. The long-term storage temperature for samples should be -80°C or lower.

In addition, any delays in sample preparations could potentially affect sample quality. Plasma separation from blood is obtained by centrifugation. A significant impact of pre-centrifugation delay on plasma protein profiles is shown in Figure 13. The delay before centrifugation of whole blood was associated with higher levels of some plasma proteins. This was interpreted as leakage of these proteins from lysed blood cells. The effects were minimal when blood was centrifuged and plasma separated within 8 hours if samples were handled at 4°C, but the protein profile is vastly altered with 1 hour at 22°C. This observation indicates that protein degradation is not the only factor to be considered in the context of temperature variations. Beyond the timelines described above, increasingly significant effects were observed on the protein levels measured. Therefore, the pre-centrifugation delay time should be kept short and consistent between samples to avoid influencing the data obtained.

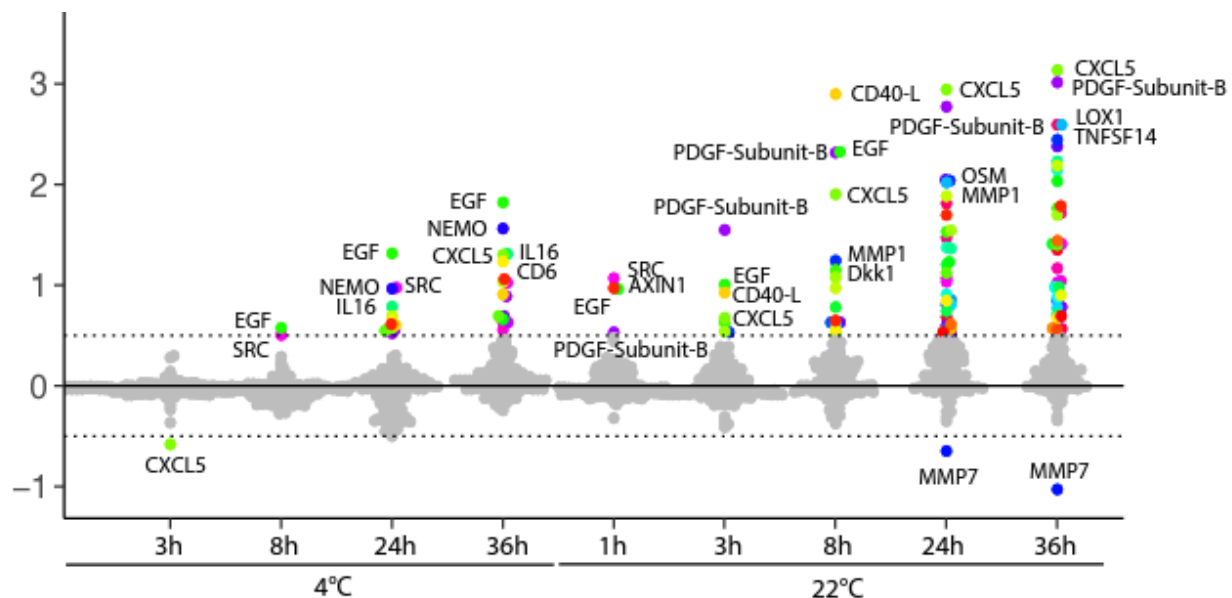


Figure 13. The impact of pre-centrifugation delay on plasma protein profiles

Freeze-thaw cycles

The effect of freeze-thaw cycles has been investigated by several research groups, and data shows that expression levels of some proteins may be affected, whereas others do not seem to be impacted. However, studies also show that the relative differences between groups remain the same for samples that have undergone the same number of freeze-thaw cycles.

The correlation of protein levels from the same samples undergoing 2 to 8 freeze-thaw cycles is shown in Figure 14 on the right. The Pearson correlation coefficient is 0.997 in these studies. This demonstrates that samples with a history of multiple freeze-thaw cycles can still be processed with high quality.

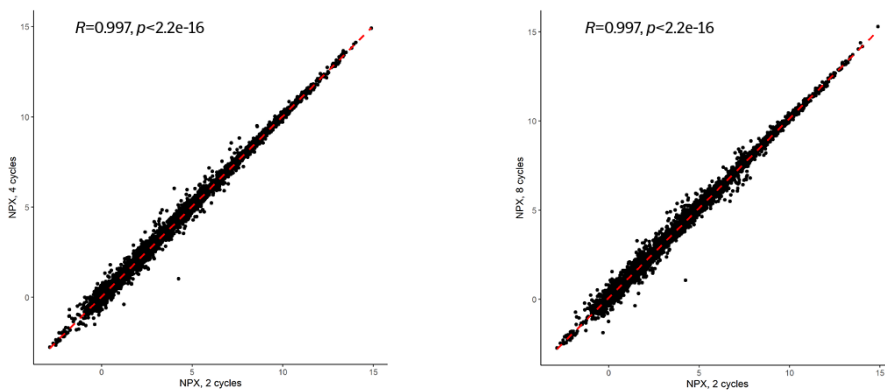


Figure 14. The correlation of protein levels from the same samples undergoing several freeze-thaw cycles

Blood components

Hemolysis

In most cases, hemolysis is an unavoidable pre-analytical effect. Hemolysis can appear due to the procedure used during blood collection, transport conditions and sample preparation. Hemolysis can lead to errors, mostly due to the leakage of cellular contents. Nevertheless, the influence of hemolysis depends on the protein measured. Levels tested for hemoglobin are 0.23 - 15 g/L. The highest concentration for the hemolysate tested translates to about 10% hemolysis (Figure 15).

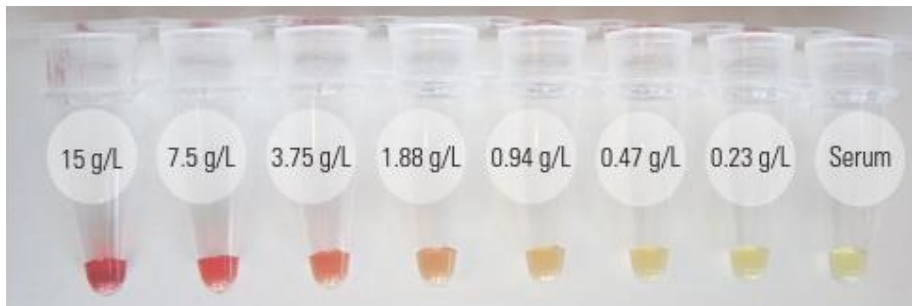


Figure 15. Different levels of hemolysis, levels tested for hemoglobin are 0.23 - 15 g/L

Other plasma components: Lipids and Bilirubin

Several plasma components are known to potentially interfere with immunoassays. In a study, the potential impacts of bilirubin and lipids were evaluated at different spiked concentrations of analytes (Figure 16 and 17). For all assays, bilirubin and lipids could be added to concentrations corresponding to at least 8 (630 mg/mL) or 10 times (20 mg/mL) normal values respectively, without affecting assay performance (data not shown).



Figure 16. Different levels of lipids, levels tested for lipids are 0.31 - 20 mg/mL

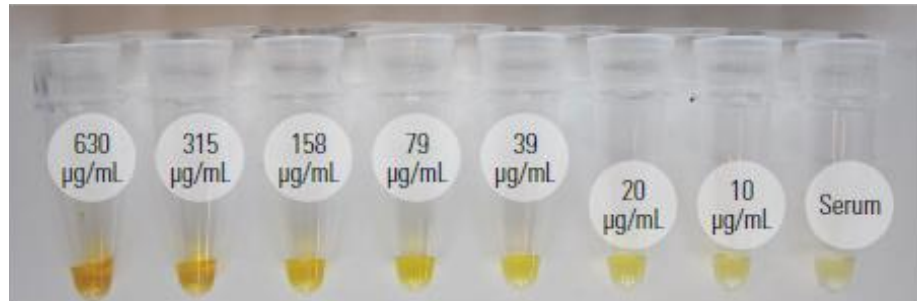


Figure 17. Different levels of bilirubin, levels tested are 10-630 µg/ml

Best practices

1. Standardize sample collection as much as possible.
2. Document how the samples have been collected and handled.
3. Store the samples at -80°C.
4. Transport samples on dry ice.
5. If possible, keep the number of freeze/thaw cycles between samples in the same range within a study.

IV. Randomization: importance of proper randomization and the best strategies

Learning objectives

- ☐ Understand the importance of randomization
- ☐ Learn the best randomization strategies

Why do we randomize a single plate?

Correct sample randomization will empower your study and minimize the risk of introducing any bias that can confound downstream analyses. If randomization is not properly performed, true biological variation may be missed or misidentified as, for example, technical variations. Randomly assigning sample groups across the plate will remove potential row and column group associations. With randomization, any significant associations within a row or column can be identified as technical variation, making the Quality Control process of your data more robust and the distinction between technical and true biological variation easier to distinguish between. An example of sample randomization on a single plate is shown in Figure 18.

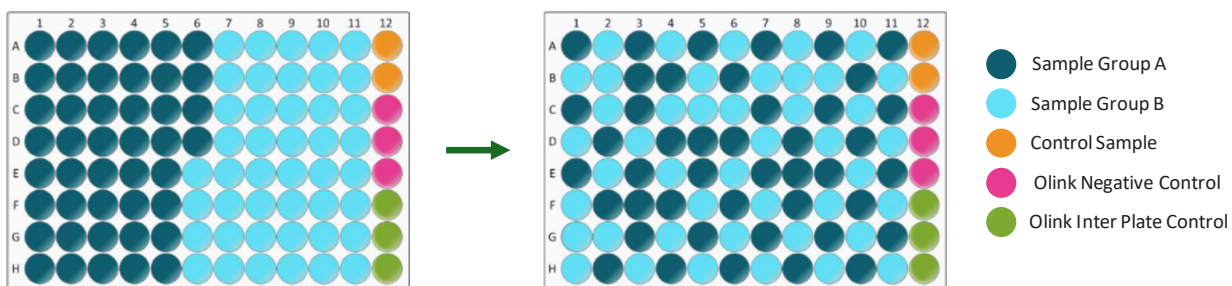


Figure 18. An example of sample randomization on a single plate

Why do we randomize multiple plates?

Unequal distribution of samples across multiple plates may remove true biological variations, as well as technical variations following normalization. An example of this is all cases on one plate and all controls on another plate. When samples are randomly assigned to plates, the plates can be considered equal, and it becomes comparable with normalization to remove technical variation. More will be explained later in Data Normalization section. An example of sample randomization on multiple plates is shown in Figure 19.

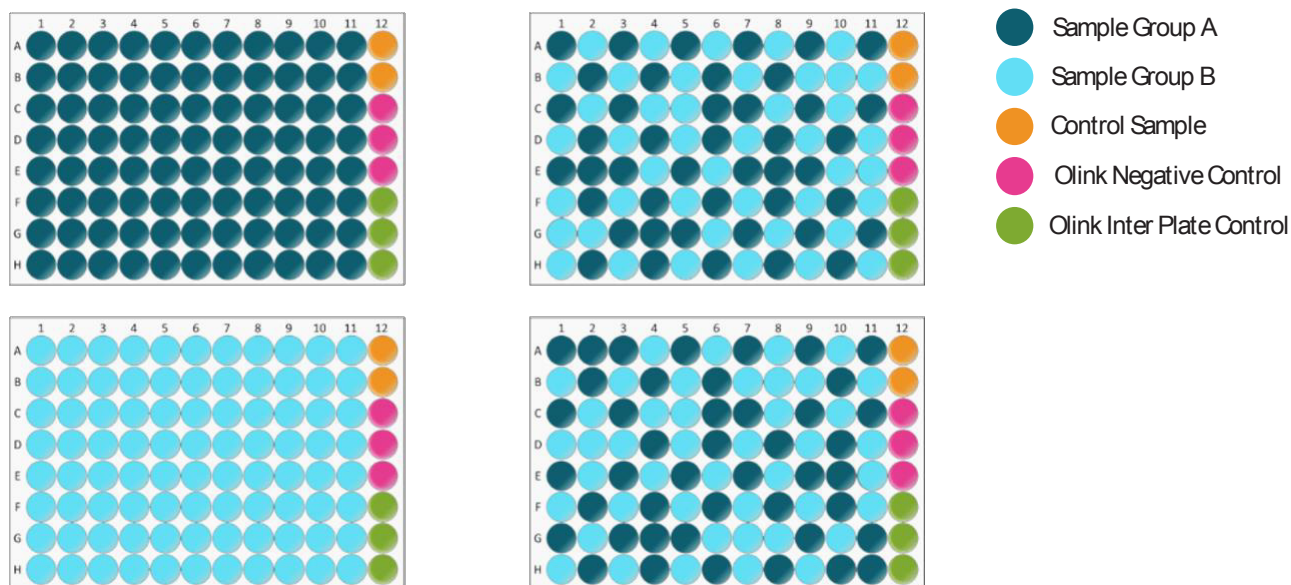


Figure 19. An example of sample randomization on multiple plates

Sample randomization methods and tools

General sample randomization methods

Commonly used methods for sample randomization:

- Randomly assigning samples to plates and wells (roll of the dice for each sample).
- A random number generator to assign samples to wells and plates.
- Olink's plate randomization manifest from our Olink® Analyze R-package.

Sample randomization for longitudinal studies

Objectives of longitudinal studies

- Primary objective: determine the change of target proteins (Δ) between different time points of the same patient.
- Secondary objective: compare the level of target proteins at a single time point between different patient groups.

Sample randomization methods

Plate all time points per patient on the same plate but randomize within and between plates.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1-1	8-1	7-5	2-1	4-1	3-1	2-2	8-3	8-4	6-2		P
B	5-4	8-5	4-2	4-4	4-4	8-1	1-2	3-5	4-1	6-3		P
C	3-1	7-3	2-3	4-3	4-2	2-3	3-2	6-3	1-5	6-1		N
D	1-3	7-3	3-2	8-2	5-3	8-3	7-2	2-2	5-1	6-2		N
E	4-5	6-4	1-3	4-5	2-1	7-3	3-4	2-2	2-5	1-5		N
F	7-5	3-3	5-2	4-3	2-5	1-4	3-4	5-2	1-4	5-4		I
G	5-1	8-2	7-2	1-1	5-5	2-4	6-1	8-4	5-3	7-1		I
H	3-3	2-4	3-5	7-1	7-4	6-4	5-5	6-5	6-5	1-2		I

Patient X- Timepoint T
Group A
Group B

P	Plasma Control
N	Negative Control
I	Inter Plate Control

Figure 20. Plate map for longitudinal study

In Figure 20, all time points of a patient should be plated on the same plate but randomized within the plate to reduce variation in the data. Highlighted in yellow, you can see five time points that are collected from each patient located within the same plate in a randomized manner. Study group A (blue) and study group B (red) are randomly distributed on the plate. Additionally, patients from different study groups are randomly distributed on the plate. Again, multiple time points from different patients within the same study group and then from different groups all being on the same plate, and each of their sample sets are randomly distributed on the plate (example highlighted in yellow and green).

For more complex studies, such as cross-sectional studies with many groups or several important covariates, a more complex randomization plan might be required. For questions or further recommendations on how to randomize your project, please contact support@olink.com.

V. What is NPX: relative quantification and how to use NPX

Learning objectives

- ☐ Learn how NPX is calculated and how to use it
- ☐ Understand relative and absolute quantification

NPX definition

NPX stands for Normalized Protein eXpression. It is a relative unit for protein quantification which is normally distributed on a log2 scale, making it suitable for downstream statistical analysis. Some key information about NPX:

NPX is an arbitrary unit on log2 scale:

- High NPX value = high protein concentration
- 1 NPX difference = 2-fold change in protein concentration

Changes in protein expression are compared by relative quantification:

- Proteins with the same NPX value may differ in actual concentration.
- Compare NPX between samples for each protein separately.
- Absolute NPX cannot be directly compared between runs.

The NPX data is ready for statistical analysis:

- Commonly used for t-tests, ANOVAs, boxplots etc.
- Comparisons between groups using NPX: the difference between e.g. Group A and Group B is best calculated by: (mean NPX of Group A) – (mean NPX of Group B).

Relative and absolute quantification

NPX is used for relative quantification and is not calculated in standard units. Rather, the relative differences in protein levels across samples are determined using the relative differences in signal readout. As shown in Figure 21, the signal in Sample 2 is 4 times higher compared to Sample 1. In the absolute quantification, the protein concentration is calculated in standard units, for example, pg/mL. This concentration is extrapolated using a standard curve generated using a reference standard with known concentrations. Both quantification methods are common in Proteomics research and each has its pros and cons. Please refer to the white paper at the end of this section for further reading.

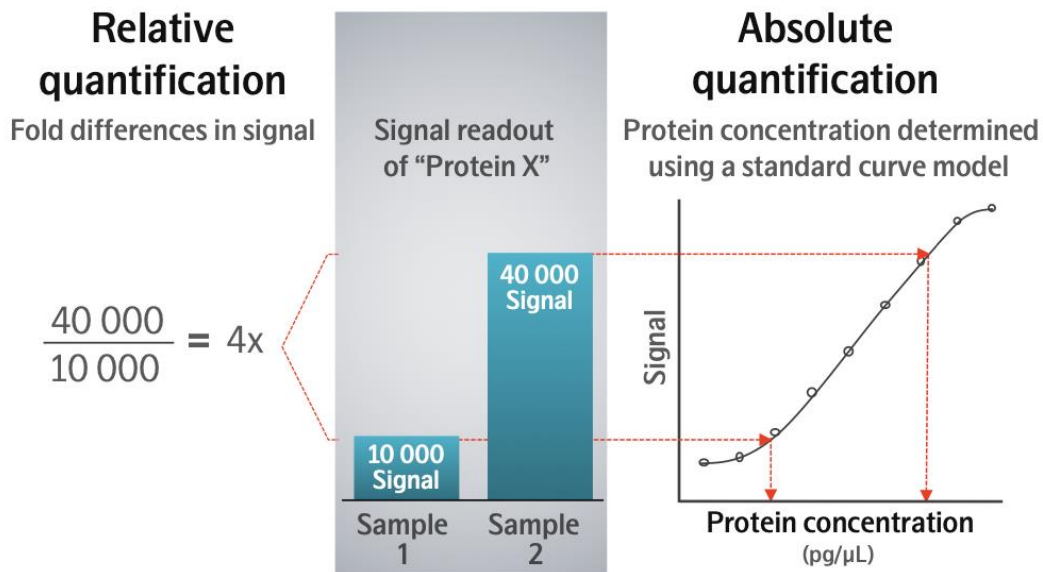


Figure 21. Absolute and relative quantification

NPX calculation

NPX calculation in Olink's relative quantification products

The NPX calculation for relative quantification is illustrated in Figure 22.

1. $Ct_{(analyte)} - Ct_{(Extension\ control)} = dCt_{(analyte)}$ *Per sample: Decreases technical variation*
2. $dCt_{(analyte)} - dCt_{(median\ IPC)} = ddCt_{(analyte)}$ *Per assay: Improves inter plate variation*
3. $Corr.\ factor_{(analyte)} - ddCt_{(analyte)} = NPX_{(analyte)}$ *Per assay: For more intuitive data*

NPX = Relative Expression Value

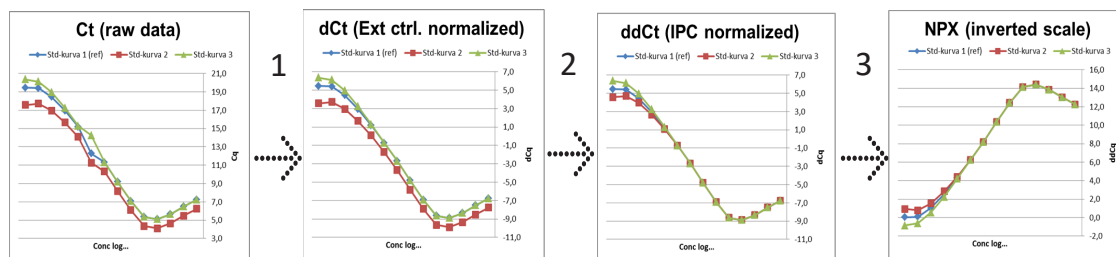


Figure 22. The workflow and NPX calculation (relative quantification)

NPX calculation in Olink's absolute quantification products

To achieve absolute quantification, the following strategies are used:

1. During product development and validation:

- A high-resolution calibrator curve (Figure 23) was established using recombinant protein and defined mathematically using a 4PL model. The linear phase of this curve determines the measuring range for each protein in the panel.
- Absolute values for calibrator and sample control are determined.

2. On each sample plate:

- Calibrator triplicates with known pg/mL for assessing inter-run variation.
- Sample control triplicates with known pg/mL for assessing accurate quantification.

In this approach, calibrators with proteins of known concentrations are run on every plate and used to align the data to the pre-defined standard curve; the performance of the calibrators is checked using sample controls with spiked-in proteins to assess the accuracy of the alignment. The samples can then be related back to the standard curve, translating the relative NPX value into the absolute amount. Unlike other immunoassays, this approach removes the need to produce a standard curve on each plate. This enables more samples to be analyzed with every Target 48 run.

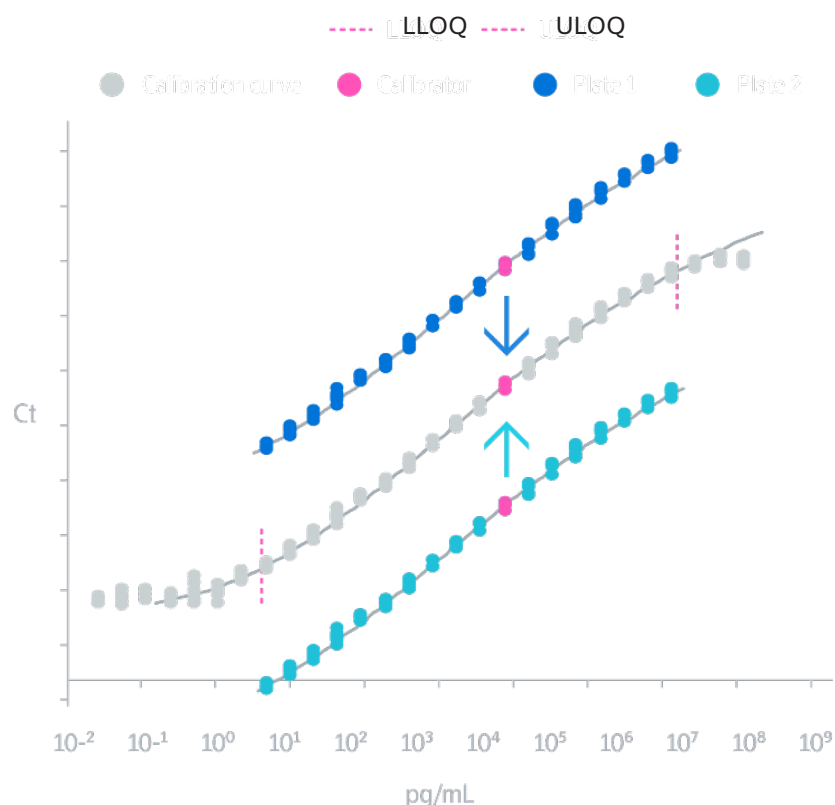


Figure 23. Calibration curve

Further reading

1. Olink® White paper: It's all relative

VI. Data normalization: appropriate normalization methods for the specific study designs

Learning objectives

- ☐ Understand the significance of proper data normalization.
- ☐ Learn about different normalization methods according to the study design.

Single plate normalization

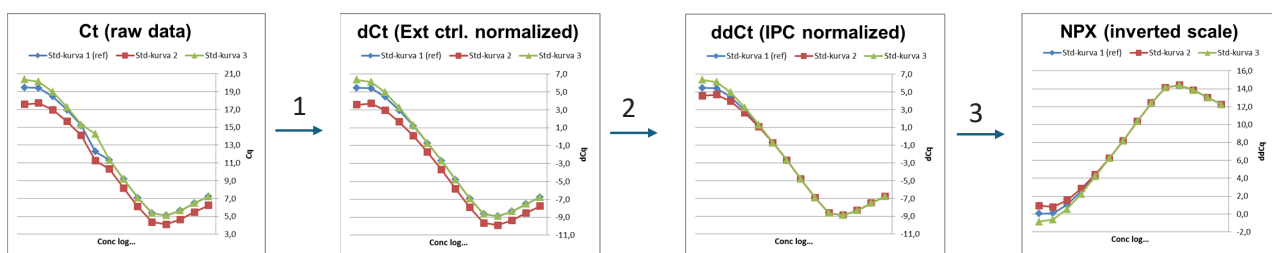
Three steps

1. Calculating NPX from raw Ct values.
2. Adjusts the NPX values based on the median interplate control (IPC) for each protein.
3. Static shift up or down for each protein.

Three benefits

1. Per sample: decrease technical variation and ensure that discrepancies in data are reflective of biological variation rather than experimental error.
2. Per assay: enhance the consistency of assay performance and improve intra-plate comparison and reproducibility.
3. More intuitive dataset: the flipped scale conversion results in a more intuitive dataset, where higher NPX values directly correlate with higher protein expression levels.

The main steps in single-plate normalization are shown in Figure 24.



1. $Ct_{(analyte)} - Ct_{(Extension\ control)} = dCt_{(analyte)}$
2. $dCt_{(analyte)} - Ct_{(median\ IPC)} = ddCt_{(analyte)}$
3. $Corr.\ factor_{(analyte)} - ddCt_{(analyte)} = NPX_{(analyte)}$

Figure 24. Main steps in single plate normalization

Multi-plate normalization

Why normalization is needed for multi-plate study

Within a single Olink PEA run, measurements of the same protein across samples on the same plate are directly comparable. However, if the same samples were run again on a different plate, there might be a shift due to variables like preparation, environmental conditions, etc. Therefore, an adjustment factor is needed to enable the comparison of values obtained on different plates, see Figure 25 and 26.

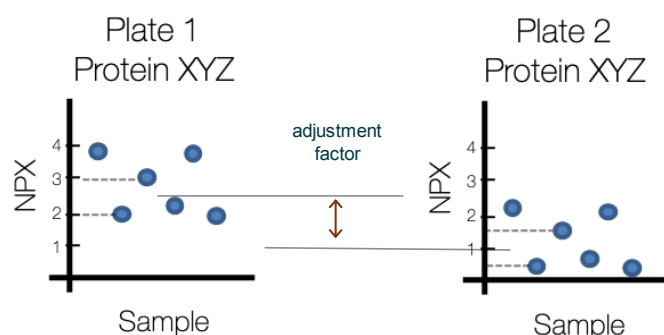


Figure 25. An adjustment factor needs to be determined to enable the comparison of data generated from different plates

The objective of normalization across multiple plates is to determine the adjustment factor for each plate to make the data comparable across plates. This factor is not a universal constant but is tailored to the specifics of each plate, accounting for the intrinsic variability in assay conditions and reagent activity. Each protein assay has a unique adjustment factor to ensure that the distinct behavior of different proteins under variable assay conditions is considered.

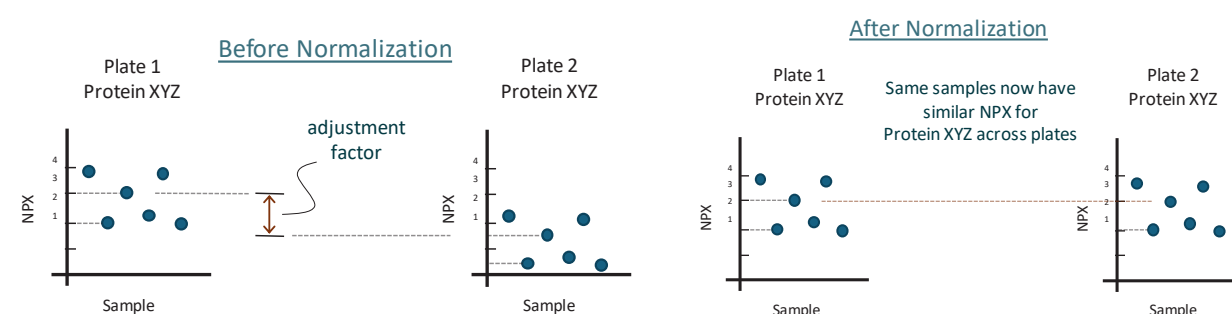


Figure 26. Adjustment factors are determined during normalization, enabling the comparison of data among multiple plates

Multi-plate normalization methods: intensity normalization and reference sample normalization

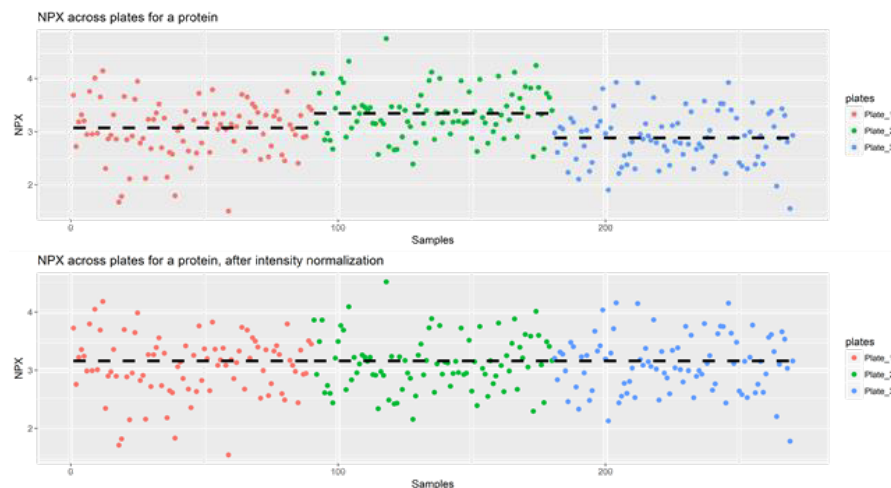
Intensity normalization

- Adjusts the NPX value for each assay so that the median of all samples is the same for all plates.
- Requires samples to be randomized across all plates in a study.

Intensity normalization improves %CV for **well-randomized studies**, see Figure 27:

- If plates can be considered randomized, small differences in intensity between plates can be safely removed for each assay.
- Plates are adjusted by calculating the median NPX value for each assay across all samples on a plate, then adjusting the median of each plate per assay to be equal.

Pre-normalization



Post-normalization

Figure 27. Intensity normalization

Reference sample normalization

- Adjusts the NPX value for each protein so that the median NPX for reference samples (also known as bridging samples) are equal.
- Apply the per plate adjustment factor for each assay to all samples.
- Requires inclusion of 8 to 24 reference samples to all plates in a study.

Reference normalization improves %CV for **non-randomized studies**, see Figure 28

If each plate contains samples from a different group, the experimental variable (plate) is mixed with the biological variable (sample group)

- Intensity normalization causes risk of removing relevant biological variation.
- Olink recommends using overlapping bridge samples (purple) instead.

Reference normalization improves the coefficient of variation for non-randomized studies. This enhancement is particularly notable in multi-batch studies where each plate contains distinct sample groups and the plate setup becomes an experimental variable.

When different plates correspond to different conditions or time points, intensity normalization may inadvertently remove meaningful biological variation. Olink advises against this, especially in such non-randomized studies, to prevent confounding the data. Instead, reference normalization is the recommended approach. By incorporating 8 to 16 for Olink® Target and 16 to 24 for Olink® Explore overlapping bridge samples, which are shown here in purple, we can achieve a more accurate normalization across multiple batches, ensuring that any variation we observe is due to the biological differences between samples rather than technical variability between plates.

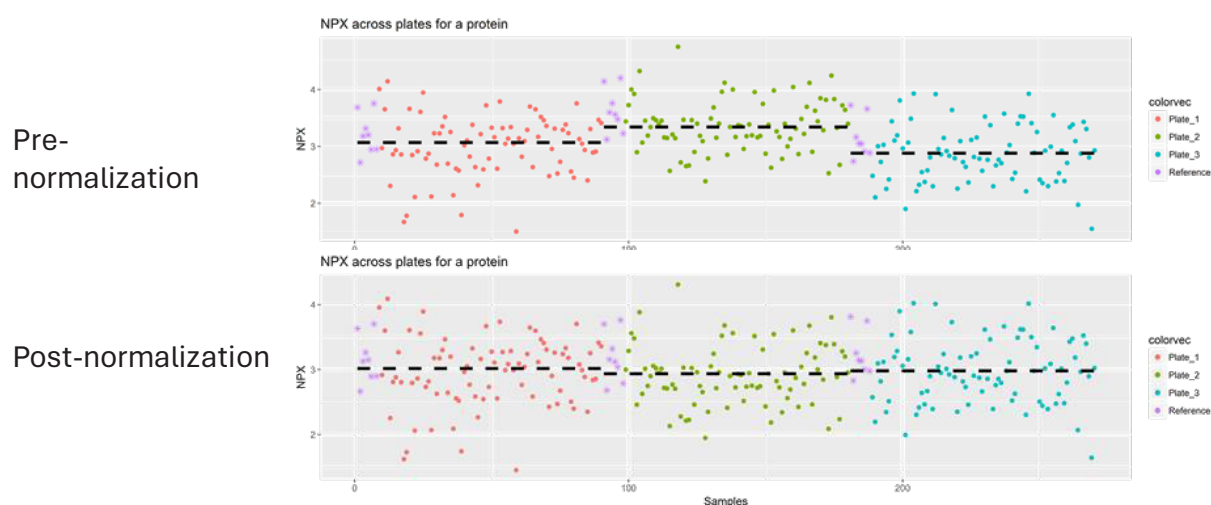


Figure 28. Reference sample normalization

Bridging (Reference) samples

Bridging samples are samples run on multiple plates or batches:

- Control for run-to-run, batch-to-batch, lot-to-lot variability.
- Run at every time point in continuous studies.
- Run on every plate of non-randomized studies.
- Used to normalize and alleviate potential bias.

How to select bridging samples:

- Use unique individual samples, do not use pooled samples
- Select samples with sufficient volume for bridging the entire study
 - If randomizing within each batch, only need to run bridging samples once per batch
 - If not randomizing, bridging samples need to run on every plate in every batch
- Select samples from all groups in the study
- We offer free tools to select appropriate bridging samples using R script in Olink analyze package (<https://github.com/Olink-Proteomics/OlinkRPackage>)

Benefits and tradeoffs of intensity normalization and reference normalization

Intensity normalization

Very effective ONLY WHEN samples are RANDOMIZED across each plate (no biases)

Reference sample normalization

- Useful when you cannot be certain of randomization between plates
 - Due to small number of samples, non-balance of samples enrolling in study, etc
 - Due to continuous study with multiple batches and potential lot changes
- Using same samples on both plates allows for direct comparisons
- TRADEOFF: More susceptible to problems if reference samples are poorly chosen, or do not accurately represent the sample set (dynamic range) of the overall study.
- TRADEOFF: Because reference sample normalization uses fewer data points, it is less robust than intensity normalization when good randomization exists across plates.

Normalization methods for studies with absolute quantification

The normalization method for studies aiming at absolute quantification (Target 48, Flex, and Focus panels) relies on calibrator normalization. This process involves the use of a calibrator, composed of pooled plasma from healthy individuals with a spike-in of recombinant antigen to establish a protein concentration baseline for the assays that fall below the limit of quantification. In this analytical framework, normalization is conducted through the calculation of NPX levels, derived from the Ct values obtained via qPCR. Each sample's NPX is individually normalized using the Extension Control. Moreover, to account for inter-assay variability, Olink provides a bridging factor, which is lot-specific, to ensure consistent and comparable quantification across different plates and batches. The use of the Extension Control and the bridging factor together minimizes potential variability, ensuring accurate and reliable quantification of proteins across the study samples.

Summary: Proteomics study design scenarios and corresponding randomization and normalization methods

The following chart summarizes important parameters to consider when designing various types of Proteomics studies (Figure 29).

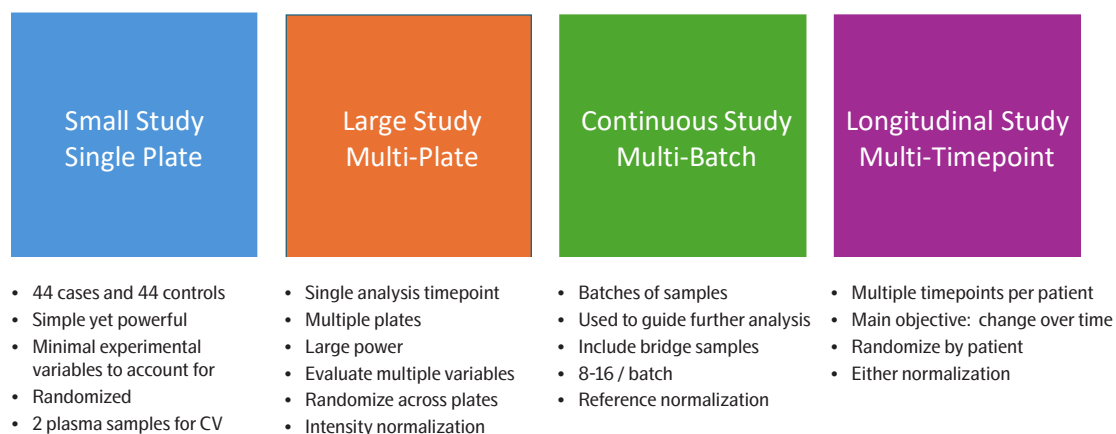


Figure 29. An overview of Proteomics study design scenarios and corresponding randomization and normalization methods

VII. Olink® toolbox for study design and data analysis

Olink® FAQs

Olink Frequently Asked Questions are grouped into categories

Olink® Insight

A free platform for protein biomarker data discovery. This is an open platform that aims to accelerate proteomics research by allowing users to see data in new ways, to collaborate faster and easier with peers, and to access exclusive data sets and data stories.

Olink® Statistical Services

Customized statistical analysis performed by experts experienced in handling Olink® data

OlinkAnalyze®

A versatile toolbox for handling of Olink® data, including QC, various statistical tests, and visualization (R Package on GitHub)

Olink® Study Size Calculator

A free web-based application for statistical power calculation (This software is available within Olink® Insight)