White paper

Data normalization and standardization

Introduction

With Olink's Proximity Extension Assay (PEA) technique, as used as in the Olink® Target 96/48 protein biomarker panels, real-time quantitative PCR (qPCR) is used in the readout step to measure relative changes in protein expression. The qPCR detects the unique DNA sequence formed when complementary oligonucleotide-tags attached to pairs of analyte-specific antibodies hybridize and extend in the presence of DNA polymerase.

Olink translates the Ct values from the qPCR into the relative quantification unit, Normalized Protein eXpression (NPX), using a series of computations. These operations are designed to minimize technical variation and improve interpretability of the results.

Olink has also developed a PEA platform with NGS readout (Olink® Explore). While the NPX calculations are different between these two readout methods, the evaluation of the data is performed in the same basic way. In this white paper, however, we will focus on normalization of data generated via qPCR readout only.

The analysis of the data can be affected by a number of technical factors. To account for this, Olink uses a quality control (QC) system that monitors the performance of assays and samples, followed by appropriate normalization that alleviates systematic noise caused by sample processing or technical variation.

Olink's built-in QC system

Olink has developed a built-in QC system, using internal controls, for its multiplex biomarker panels. This system enables full control over the technical performance of assays and samples.

Internal controls

The QC system consists of four internal controls that are spiked into every sample and are designed to monitor the three main steps of the Olink protocol: Immunoreaction, extension and amplification/detection (Figure 1).

Incubation controls: Incubation Control 1 and 2 are two different non-human antigens measured with PEA. These controls monitor 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 immunoreaction. This control monitors variation in the extension and amplification/detection step and is used to adjust the signal from each sample with respect to extension and amplification.

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.

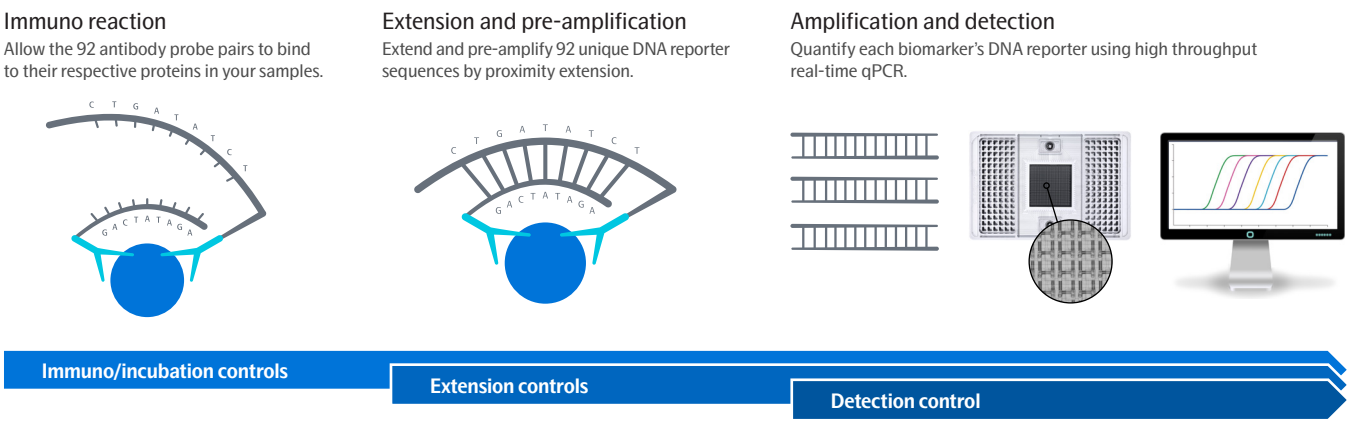

Fig 1. Main steps and controls in a PEA assay with qPCR readout.

Sample controls

There are six required and two recommended external controls that are added to separate wells on the plate.

- **Inter-plate controls**
  Inter-plate Control (IPC) is included in triplicate on each plate and these are run as normal samples. The IPC is a pool of 92 antibodies, each with one pair of unique DNA-tags positioned in fixed proximity and can be seen as a synthetic sample, expected to give a high signal for all assays. The median of the IPC triplicates is used to normalize each assay, to compensate for potential variation between runs and plates.
- **Negative controls**
  Negative Control is also included in triplicate on each plate and consists of buffer run as a normal sample. 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.
- **Sample controls**
  It is highly recommended to include a pooled plasma sample, run in duplicate, on each plate. This sample control will be used to assess potential variation between and within plates through the calculation of inter- and intra-assay CV's.

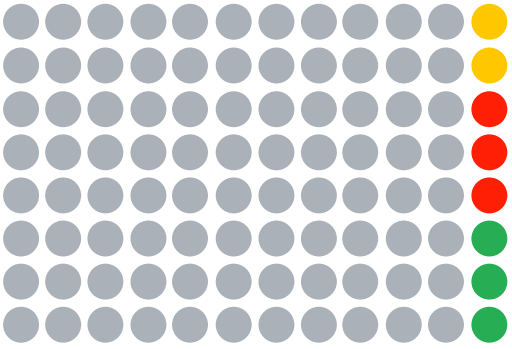

Fig 2. Recommended control setup for a PEA run.

Sample QC

Each of the internal controls is spiked into the samples at the same concentration. The signals for these are therefore expected to be the same over the plate. Sample QC is performed using the Detection Control and Incubation Control 2, on the initially calculated NPX values. Within each plate, the levels of these controls are monitored for each sample and compared against the median of all samples. If either of the controls differ by more than the acceptance criteria ±0.3 NPX, the sample is considered deviant and fails QC. Deviating values for the internal controls can be caused by factors such as errors in pipetting or pre-analytical variations in the samples that affect the performance, for example matrix effects.

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Matrix effects are defined as changes in the analytical readout that can be caused by all other sample components except the specific analyte to be quantified.

Plate QC

The internal controls are also used in plate QC. This assesses the variation over the plate for each of Incubation Control 1 and 2 and the Detection Control. If the variation for one of the controls is too large, the entire plate is considered unreliable.

Normalization using inter-plate controls

Calculation of NPX

Olink uses an arbitrary, relative quantification unit called Normalized Protein Expression (NPX). In qPCR, the x-axis value of the point where the reaction curve intersects the threshold line is called the Ct, or "threshold cycle." This indicates the number of cycles needed for the signal to surpass the fluorescent signal threshold line. NPX is derived from the Ct values obtained from the qPCR using the following equations:

Extension Control:

$$Ct_{Analyte} - Ct_{Extension\ Control} = dCt_{Analyte}$$

Inter-plate Control:

$$dCt_{Analyte} - dCt_{Inter\text{-}plate\ Control} = ddCt_{Analyte}$$

Adjustment against a correction factor:

$$Correction\ factor - ddCt_{Analyte} = NPX_{Analyte}$$

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The **correction factor** is calculated by Olink during the validation of the panels. The value is pre-determined, using Negative Control, and used to invert the scale so that a higher value corresponds to a higher signal. It is also used to ensure that background levels are approximately zero.

NPX is a relative quantification unit logarithmically related to protein concentration. Even if two different proteins have the same NPX values, their absolute concentrations may differ. NPX should be compared for each assay separately between samples within a run. NPX should not be compared between runs without proper inter-plate normalization due to the risk of falsely interpreting shifts in median between runs as a biological difference. However, relative differences in NPX can be compared more easily, often without additional normalization steps.

Example curves

Raw data

Below is an example of raw data for three runs with a calibrator curve. There are deviating samples in the middle, and one curve has a parallel shift.

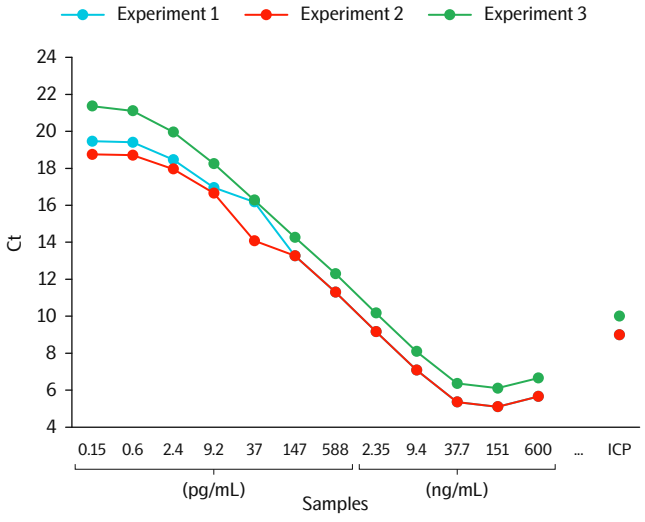

Fig 3. Raw data for three runs.

Adjusted against Extension Control

The raw data is then adjusted against the Extension Control per sample to improve intra-assay repeatability by reducing technical variation introduced in the extension step.

$$Ct_{Analyte} - Ct_{Extension\ Control} = dCt_{Analyte}$$

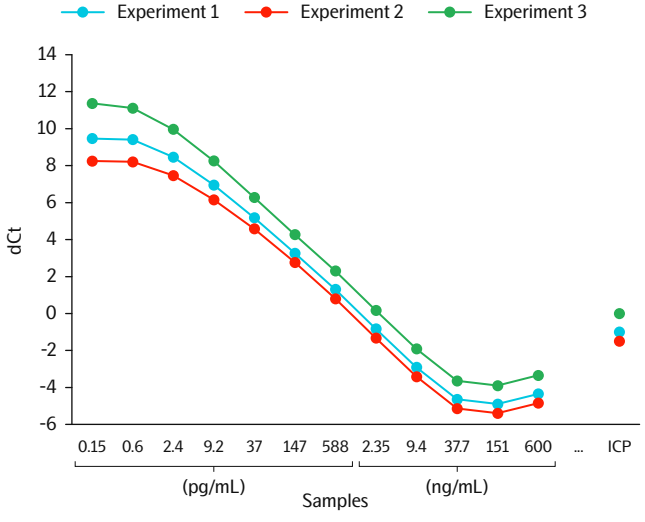

Fig 4. Adjusted against Extension Control.

Normalized against IPCs

To improve inter-assay repeatability, the data is then normalized against the IPCs. This is done per assay and plate and may be followed by intensity or reference sample normalization depending on the study characteristics.

$$dCt_{Analyte} - dCt_{Inter\text{-}plate\ Control} = ddCt$$

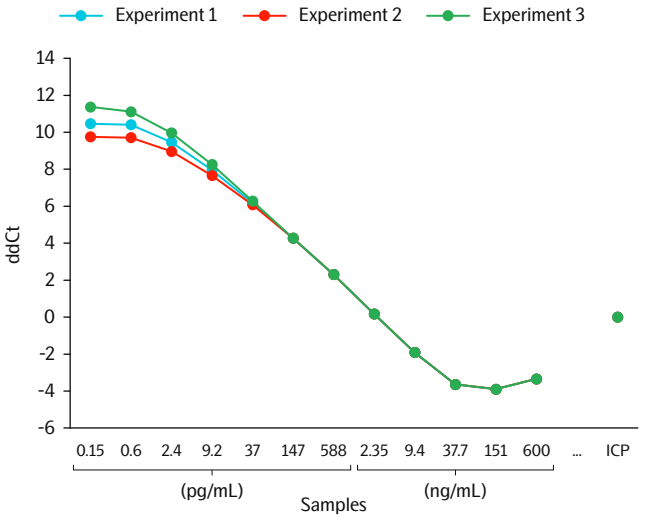

Fig 5. Adjusted against IPCs.

Adjusted against correction factor

The curve is then inverted so that a high NPX value means high protein concentration which allows more intuitive interpretation. A difference of 1 NPX approximates to a doubling of the protein concentration regardless of protein.

$$Correction\ factor - ddCt = NPX$$

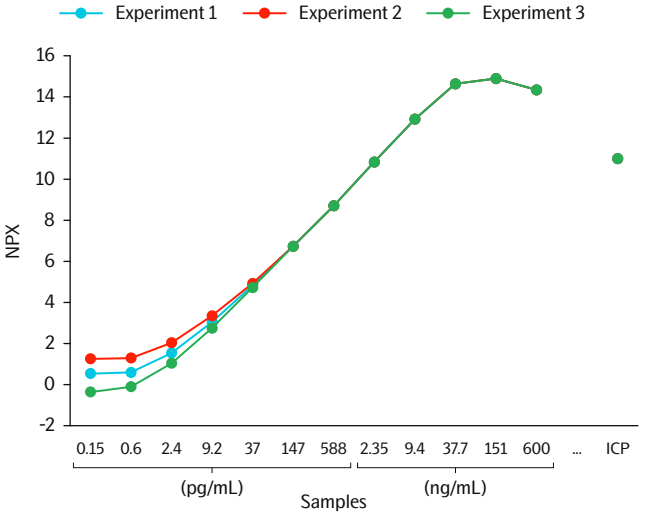

Fig 6. Adjusted against correction factor.

Inter-plate normalization methods

Olink recommends one of two normalization methods depending on the study design. For randomized studies, IPC normalization should be replaced with intensity normalization, which will increase statistical power and reduce technical variation between plates and projects. Intensity normalization can also be used when combining studies from different sources as long as the sample distribution can be considered comparable between the data sets.

For non-randomized studies, Olink recommends reference sample normalization. Running reference samples on all plates is a good strategy to minimize technical variation. When applied correctly, both intensity normalization and reference sample normalization can increase the power in a given study by reducing technical variation, since they are based on real samples in contrast to the IPC samples.

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Randomization in this context applies to the sample placement across the plates. A sample set is randomized if the relevant experimental variables can be considered evenly distributed across plates. The variables to consider must be decided for each study. They can for example include study groups, treatment, time points or demographics. If the randomization is not appropriate with regards to experimental variables, the normalization might remove true biological variation that otherwise could have been identified.

In studies where samples are randomized across plates, a global adjustment is used to centre the values for each assay around its median and across all plates. This is called intensity normalization. If randomization is not feasible or cannot be guaranteed, Olink recommends including reference samples that are representative of the cohort. For example, pooled plasma samples can be included on all plates to ensure maximum control over any systematic biases. This method also allows for improved handling of technical variation when combining studies.

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Bias can be defined as a signal attributed to experimental, biological or technical aspects that causes a systematic error. Measured protein expression levels can be affected by several sources of bias including pre-analytical and technical variation, but also incorrect sampling. Normalization can reduce introduced biases if properly implemented. Advice on sample handling and processing is provided in Olink's white paper Pre-analytical variation in protein biomarker research (www.olink.com/downloads).

Intensity normalization method

The assumption behind intensity normalization is that on average, there is no expected difference between the median signal for an assay on one plate compared to another. If any such difference is seen between plates, it can be construed as technical bias and be safely removed, resulting in more comparable values.

Olink uses intensity normalization, with the median as the normalization factor, when samples are randomized across plates and projects. The intensity normalization adjusts the data so that the median value for an assay on each plate is equal to the overall median across all plates. This method assumes that the actual median of each plate is the same. The way to ensure this is to randomize the samples beforehand. If complete randomization can be assumed, this is a robust and high-performing normalization method. Intensity normalization is performed in the following way:

Step	Description
1	For each assay, calculate the overall median value for all samples and plates.
2	For each plate and assay, calculate the plate specific median value.
3	For each assay, subtract the plate specific median from every value for the plates (equals centralizing to median 0).
4	For each assay, add the overall median value (equals centralizing to the overall median).

Reference sample normalization method

When samples are not randomized across plates, the inclusion of eight or more bridging reference samples on each plate can be used for normalization. Reference sample normalization is performed in the following way:

Step	Description
1	Choose a reference plate to normalize towards.
2	For each assay and plate, calculate the pairwise difference for each of the overlapping samples with the reference plate.
3	Estimate the plate- and assay-specific normalization factor by calculating the median for the pairwise differences calculated in step 2.
4	For each assay and plate, add the plate- and assay-specific normalization factor from step 3 to each value, to normalize it to the reference plate chosen in step 1.

Evaluation

To evaluate how effective a normalization method is, Olink investigates the NPX distributions and compares the average %CV for different normalization methods.

Intensity normalization evaluation

The following illustration depicts the NPX of a protein across three different plates, where the samples have been randomized. The colors indicate different plates.

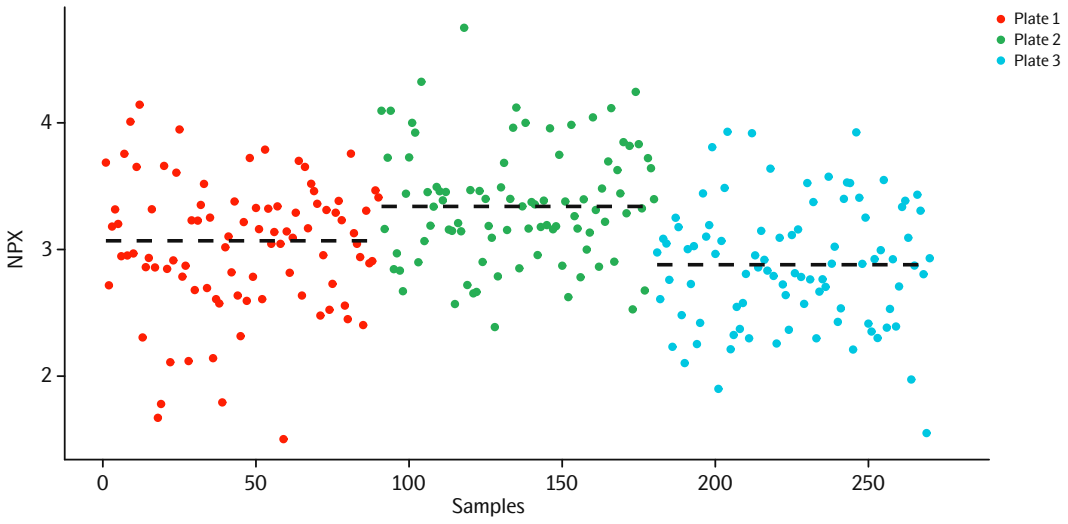

Fig 7. NPX across plates for each protein.

After intensity normalization the median NPX is leveled.

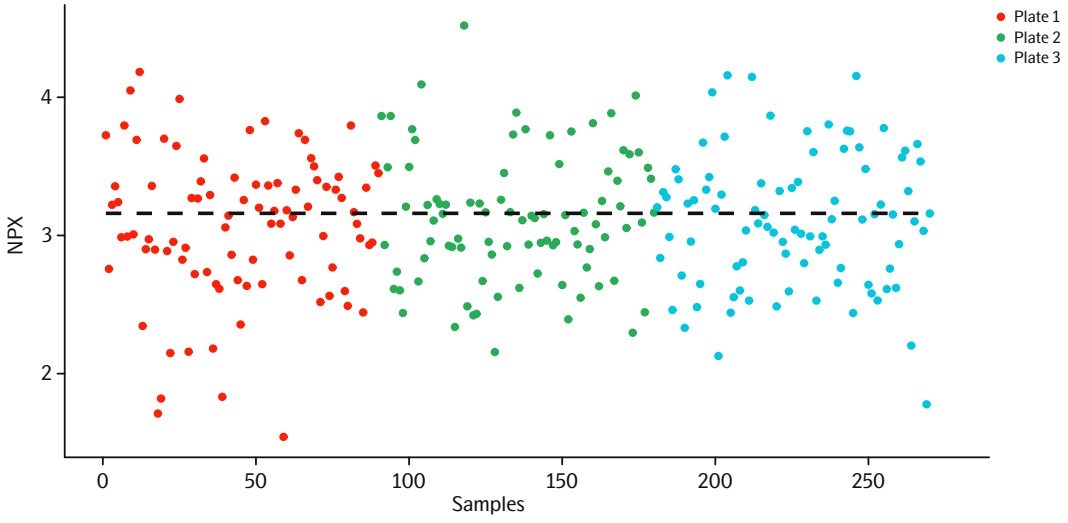

Fig 8. NPX across plates for each protein after intensity normalization.

In properly randomized studies, intensity normalization typically improves average %CV by a few percent.

Reference sample normalization evaluation

In the following example, the samples are poorly randomized, and the plate in the middle contains mostly samples from one group. This means that technical variation between plates is mixed with the biological variation that can be investigated by comparing the sample groups. In this case, intensity normalization of the plates would remove some of the relevant biological variation when correcting for technical variation between plates. The illustration shows the NPX values of the proteins in poorly randomized samples and the NPX values of the reference samples. The reference samples are shown in purple.

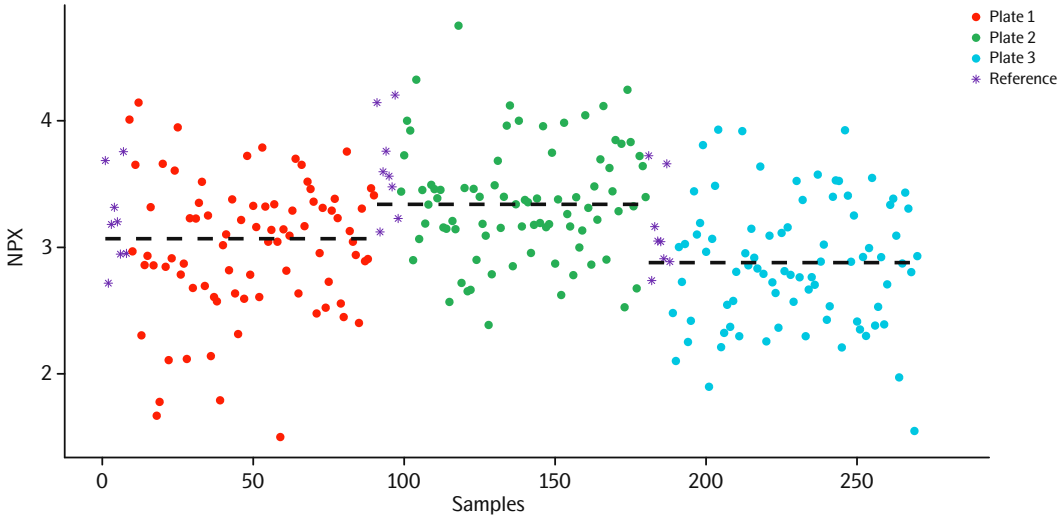

Fig 9. NPX across plates for each protein including reference samples.

The reference samples are then used for normalization. After reference sample normalization, the median NPX can still differ between plates, reflecting different compositions of sample groups.

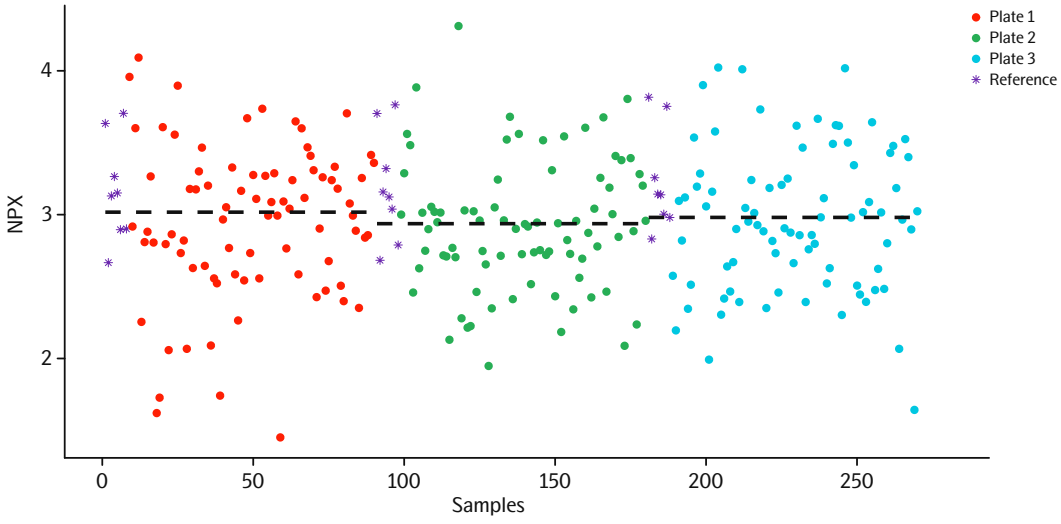

Fig 10. NPX across plates for each protein after reference sample normalization.

Misinterpreted data

To further illustrate issues with improper normalization, the image below gives an example of the bias introduced into a dataset when normalization factors are driven by variance introduced by biological factors, and not simply by inappropriate sample preparation or instrument-based variability. The illustration shows a poorly randomized set of samples where the difference between the two groups is statistically significant (p<0.05). In this case, intensity normalization would remove that difference.

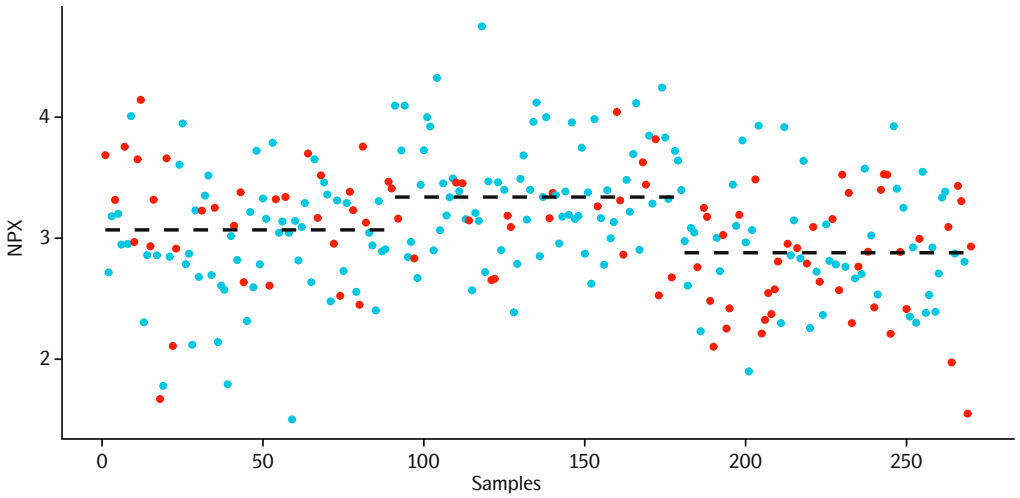

Fig 11. NPX across plates for each protein, poorly randomized samples.

The second plate contains samples from mainly one group. Here the technical variable (plate) is mixed with the biological variable (sample group), and intensity normalization cannot separate between the two, as shown in the following image.

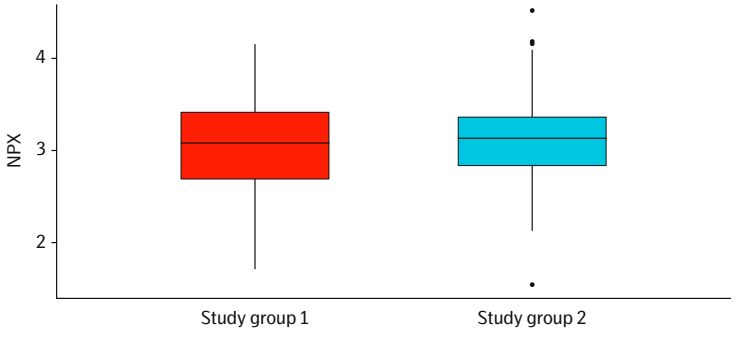

Fig 12. Intensity normalization removed the difference (p = 0.52).

With reference normalization, it is possible to see a significant difference.

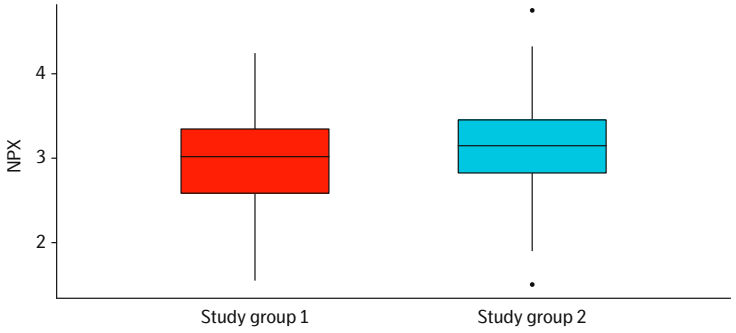

Fig 13. Significant difference with reference sample normalization (p<0.05).

Olink can help

Normalization is important to remove systematic variation, but needs to be applied carefully to minimize the risk of removing true biological variation. As a service, Olink can discuss normalization approaches and study design with you before analysis starts.

If you send your samples to Olink's Analysis Service team, the data analysis steps will be taken care of. They will provide you with a comprehensive NPX data and QC report after your study is completed.

If more extensive data analysis services are required, our biostatisticians in the Olink Data Science team can help you with customized statistical analysis.

Olink's Data Science offering currently includes

- **Olink® Insights Stat Analysis**
  A free web-based application for basic data visualizations and statistical analyses (Shiny App).
  www.olink.com/biostat-apps
- **OlinkAnalyze (RPackage on GitHub)**
  A versatile toolbox for handling of Olink data including QC plot functions, normalization, various statistical tests and modelling.
  www.olink.com/biostat-apps
- **Olink Statistical Services**
  Performed by experts experienced in handling Olink data.
  www.olink.com/biostat-services

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