White paper

Pre-analytical variation in protein biomarker research

Introduction

Proteins are a critically important class of biomolecules, since they directly control and regulate most of the body's biological functions. They are crucial for health and disease, and the drugs we currently have available largely exert their effects via interactions with proteins. Unlike the genome, the proteome is very dynamic and changes significantly in response to biological signals, environmental conditions and other external stimuli. Given this central role, proteins can serve as invaluable biomarkers to better understand biological mechanisms and to monitor health, therapy effects or the real-time status of any given disease.

Compared to genes, which have been extensively used as biomarkers, proteins are less stable than DNA and therefore require more care in their storage and handling. Taking an extreme example, the DNA of a dinosaur can provide sequence data millions of years after it would have been possible to carry out any reliable analysis of its proteins (Fig. 1).

Figure 1. DNA vs proteins. You can sequence the DNA in food long after you can't eat it anymore (but storage at the right temperature helps with the latter).

Olink Proteomics has developed high-quality immunoassays for protein biomarker research that can measure many different proteins simultaneously. But to ensure the best possible data, this assay performance needs to be matched by good quality, properly handled samples. Proteins can be affected by several factors related to collection and storage, all of which need to be considered when measuring proteins in biological samples and interpreting results. It is important to document as much information as possible about the samples and how they have been collected. Taking this approach, it is perfectly possible to obtain meaningful results even with samples that have not been handled optimally. Proper documentation around sample handling can also provide valuable input when interpreting the data.

This white paper will provide some guidance on what to consider with respect to sample collection and handling when setting up a study. This advice is generally applicable to protein studies using any immunoassay-based approach and is not specific for Olink's technology.

Serum and plasma

Olink panels can be used for a wide range of sample matrices (see olink.com/knowledge/faq for details), but this document will focus on blood samples. Plasma and serum are both derived from whole blood but a difference in measured levels can be expected for many proteins. In a study by Rohini Rajan *et al.* (1) the importance of carefully choosing between plasma and serum for biomarker studies is highlighted, as this can affect the detectability and sensitivity of biomarkers, potentially influencing the conclusions drawn from the research. Their findings suggest that while many biomarkers are consistent across both biofluids, specific markers require careful consideration of the sample matrix used.

Serum

Serum is the liquid part of blood after it has been allowed to coagulate fully for 30-60 minutes at room temperature. Serum is free of clotting proteins but contains the clotting metabolites that result from the clotting process. The clot is removed by centrifugation. There are two common types of collection tubes for serum, with (yellow lid) and without (red lid) gel separator. Tubes with gel separator are referred to as serum separator tube (SST). The gel forms a physical barrier between the clot and the serum and contains particles that cause the blood to clot quickly.

Plasma

Plasma is the liquid part of blood that has been treated with anti-coagulants, after cells have been removed by centrifugation. Since plasma has been prevented from clotting it is reflective of the blood as it circulates in the body. Plasma collection tubes contain different anti-coagulants such as EDTA, heparin, or sodium citrate, and any of these additives can be used in Olink's analysis, but for consistency, all samples within a study should be collected the same way.

Pre-analytical factors that affect protein biomarker studies

Standardization of sampling procedures is important. The key in any analysis is that the case and control samples are handled consistently throughout the entire analytical process from study design and collection of samples to data analysis. However, this is not always possible. When multiple labs are involved or when biobank samples with uncertain history are used, several factors can affect the quality.

Interference from blood components

In most cases, hemolysis is an unavoidable pre-analytical effect. It can appear as a result of the procedure used during blood sample collection and also due to transport conditions and sample preparation. Hemolysis can lead to errors in many common determinations in clinical chemistry, mostly due to the leakage of cellular contents. Nevertheless, the influence of hemolysis depends on the protein measured. Bilirubin and lipids are other blood components that can interfere with immunoassays.

The potential impact of hemolysate is evaluated by using serial dilutions in EDTA plasma and serum (Fig. 2).

In order to provide guidance on potential interference issues, the highest concentration of hemolysate that does not have an impact on assay performance is documented for each biomarker in our panels. This information can be found in the Validation Data document for each panel.

Hemolysate

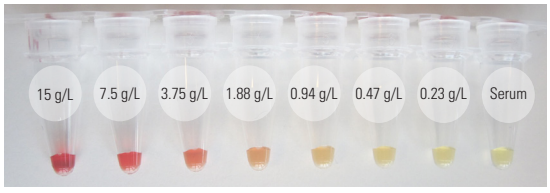

Figure 2. Endogenous interference. Levels tested for hemoglobin are 0.23 – 15 g/L. The highest concentration translates to about 10% hemolysis.

A collection technique that prevents cell lysis could greatly improve sample stability. Cell-free DNA blood collection tubes are gaining recognition in the field as for example described by Almazi *et al.* (2).

Temperature

Temperature is a major variable in protein handling. Everything from temperature during handling process and transport to long-term storage temperature has impact on the quality of the samples. Protein stability and enzyme activity are temperature-dependent. Protein degradation can be reduced by keeping samples cold during handling and transportation, and cold storage can prevent leakage of cellular proteins. Olink's customers are asked to deliver samples on dry ice to minimize the degradation of proteins. The long-term storage temperature for samples should be -80°C or lower.

Plasma sample preparation

Plasma separation from blood is obtained by centrifugation. A significant impact of precentrifugation delay on plasma protein profiles has been shown in a study performed by Shen *et al.* (3). They revealed a general trend that a longer time of delay before centrifugation of whole blood was associated with higher levels of some plasma proteins (Fig. 3). 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 increases were seen already after just 1 hour if handled at room temperature (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 precentrifugation delay time should be kept short and consistent between samples to avoid influencing the data obtained.

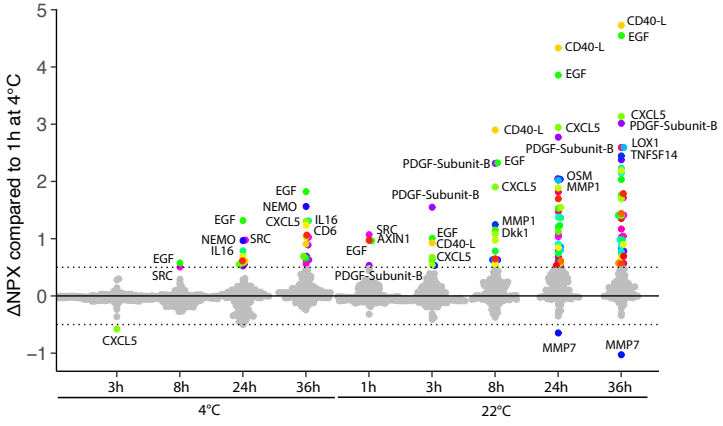

Figure 3. Alterations of protein levels. Demonstration of protein level changes at each delayed time point at 4°C and 22°C. The ten proteins whose levels altered the most are highlighted.

Plasma protein levels after delayed centrifugation times have also been investigated in another study performed by Huang *et al.* (4) They showed that plasma protein levels were significantly affected over a range of delayed centrifugation times from 1 to 72 hours, which correspond to common postal transit delays.

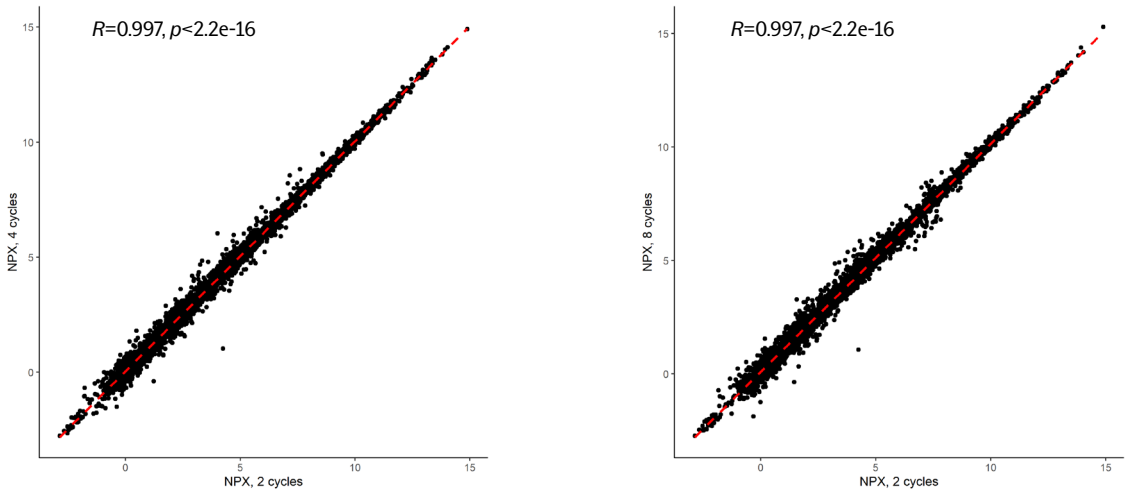

Figure 4. Effect of repeated freeze-thaw cycles on protein profiles in plasma. Demonstration of the correlations of protein levels in samples from 16 individuals that had been subjected to two freeze-thaw cycles compared to four (left plot) or eight (right plot) cycles and analysed on five Olink panels (Olink in-house data). The Pearson correlation coefficient (R) equals 0.997 in both cases. Line of equality (y = x) is shown as a dotted red line. Each correlation panel includes data for 16 individuals x 460 proteins= 7360 data points.

They also demonstrated that it is possible to predict the centrifugation delay time based on changes in protein levels, showing the value of using biomarkers for detecting pre-analytical variation.

Long-term storage and freeze-thaw cycles

The quality of biobank samples is important for protein biomarker research. Biobanks should strive to maintain the sample composition at the time of collection, but long-term storage is also a pre-analytical factor to consider. In a study by Enroth *et al.* (5) where they looked at how protein levels change with age of individuals, sample storage time was examined as an independent variable. The analysis was performed on samples from 50 year old individuals, where the samples had been stored for 30 years at -80°C. Eighteen of the 108 investigated proteins were influenced by storage time, and among these 18 proteins, the storage time alone explained between 5 to 35% of the variation seen for a single protein.

The joint analysis of two or more Olink datasets using relative quantification (NPX), requires normalization using bridging samples that are analyzed in both datasets.

Long-term storage of bridging samples has been investigated by Christensen *et al.* (6), and in their study they show that bridging samples could be stored for over three years and be used on different versions of the same Olink panel without results being affected, enabling researchers to compare the results obtained in different studies.

It is also important to be aware of how different components behave during repeated freeze-thaw cycles to be able to use the limited biobank material from one individual to its maximum potential. The effect of freeze-thaw cycles has been investigated by several research groups (e.g. Lee *et al.* (7)) and data shows that expression levels of some proteins may be affected whereas others do not seem to be impacted. However, studies (e.g. Shen *et al.* (3)) 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 several freeze-thaw cycles have been investigated by Shen *et al.* as well as in an Olink in house study (Fig. 4). The Pearson correlation coefficients were 0.998 and 0.997 respectively in these studies. Thus demonstrating that samples with a history of multiple freeze thaw cycles can still be processed with high quality.

Given the challenges of data collection and sample storage within particular studies, there has been little standardization across biobanks. A full data trail on each sample should be provided by the biobank to enable the samples to contribute as part of wider collaborative efforts with other similar samples.

Sample collection

There is a need for careful matching of pre-analytical conditions of samples collected from cases and controls. For example anasthesia and fasting before a surgery can alter the plasma proteome, as shown in a study by Gyllensten *et al.* (8) They found that 421 out of 983 proteins (42.8%) showed statistically significant differences in plasma abundance levels when comparing samples collected from awake patients at the time of diagnosis and samples collected before surgery when patients were under general anaesthesia.

The recommendation is to collect plasma samples for bio-banking from awake, non-fasting patients to minimize bias when comparing with healthy controls or other study populations.

Transportation

Potential temperature issues arise in the transportation of samples within and between facilities. Care must be taken to ensure that the serum and plasma samples have sufficient dry ice for the expected duration of the transport.

Future developments: Filter papers and dried blood spots

Liquid samples are still the most common type for biomarker research, but filter paper samples with for example dried blood spots (DBS), plasma, tear fluids or vaginal fluid are attractive because of the ease and low cost of collection and storage.

One study performed by Berglund *et al.* (9) demonstrated the feasibility of measuring proteins from vaginal fluid and plasma dried on filter papers. Another study, performed by Björkesten *et al.* (10), investigated the suitability of DBS, stored at −24°C, for protein measurements. The main findings from the latter were that the act of drying only slightly influenced detection of blood proteins, even after storage for 30 years.

How Olink can help

Even with the best intentions and preparations, variations can occur pre-analytically, resulting in individual outliers or overall drift. Olink's technical support can guide you on these matters, and our data science team can help with data analysis. The more information you can provide regarding sample collection, preparation and storage, the better the chances for our data scientists to identify potential problems to be able to normalize data between samples with different pre-analytical histories.

Best practice

- Newly collected plasma samples: Centrifugate plasma samples as soon as possible, but at least within 1 hour at room temperature or within 8 hours if the samples are kept at 4°C.
- Newly collected serum samples: Allow serum to fully clot for 30-60 minutes at room temperature prior to centrifugation.
- Randomize samples and always analyze the same kind of sample types as cases and controls.
- Minimize subject-related variation by standardizing sampling conditions (e.g., fasting status, time of day).
- Use dry ice for transportation.
- Store samples in a -80°C freezer, as recommended by the global biobanking organization ISBER (11).
- Document how the samples have been collected and handled.
- Keep sample handling as consistent as possible for all samples in a study. For example:
  - Keep temperature and centrifugation conditions for plasma collection the same for all samples.
  - Keep clotting time and conditions the same for serum collection.
  - Use the same type of collection tubes (important both for serum and plasma samples).
  - If possible, try to keep the number of freeze thaw cycles in the same range for all samples.

References

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