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.

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. To ensure the best possible data, however, 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 sample and how it has 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 www.olink.com/sample-types 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.

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.

Plasma

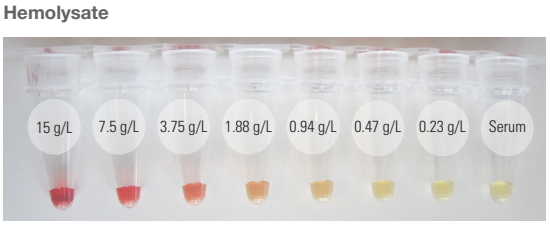
Plasma is the liquid part of blood that has been treated with anti-coagulants, after cells have been removed by centrifugation. Since plasma has been prevented from clotting it is reflective of the blood as it circulates in the body. Plasma collection tubes contain different anti-coagulants such as EDTA, 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 treated 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 multilple 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. Interference by these components in Olink assays, however, has typically only been observed at extreme levels corresponding to 8 or 10 times normal values, and are therefore unlikely to be significant factors. The potential impact of hemolysate is evaluated by using serial dilutions in EDTA plasma and serum.



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

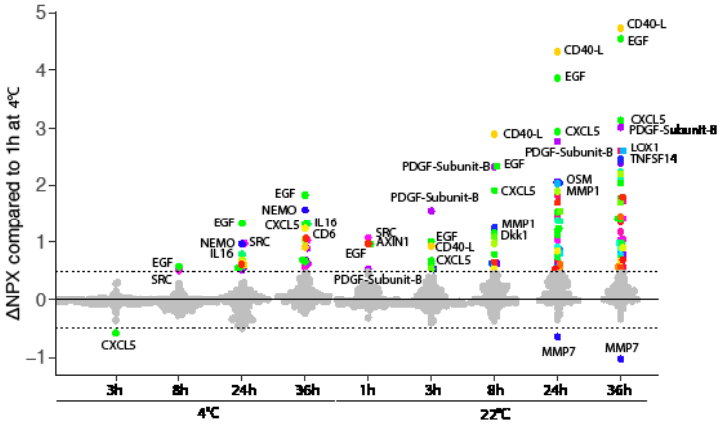
In order to provide guidance on potential interference issues, the highest concentration of hemolysate that does not have an impact on assay performance is documented for each biomarker in our panels. This information can be found in the Validation Data document for each panel.

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. (1). 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. 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.



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.

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 S. et al. where they looked at how protein levels change with age of individuals, sample storage time was examined as an independent variable. The maximum protein variation shown was in samples from 50 year old individuals, where the samples had been stored for 30 years at -80°C. In this case, storage time alone accounted for up to 35% of the variation seen in a single protein (2).

It is important to know 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. To assess the effect of multiple freeze-thaw cycles, Shen et al. used Olink assays to detect protein levels in plasma after two, four and eight

cycles. No significant change among the measured proteins was observed after eight freezing cycles, which indicates that protein levels are stable, and samples with a history of multiple freeze-thaw cycles can still be processed with high quality (1).

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.

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

Liquid samples are still the most common type for biomarker research, but filter paper samples with for example dried blood spots, plasma, tear fluids or vaginal fluid are attractive because of the ease and low cost of collection and storage. One study performed by Berglund M. et al. demonstrated the feasibility of measuring proteins from vaginal fluid and plasma dried on filter papers (3). Another study, performed by Björkesten et al., investigated the suitability of dried blood spots, 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 (4). Dried blood spot biobanks could prove of great medical value by enhancing discovery as well as routine analysis of blood biomarkers.

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
- Use dry ice for transportation
- Store samples in a -80°C freezer, as recommended by the global biobanking organization ISBER (5)
- Document how the samples have been collected and handled

References

1. Shen et al., Strong impact on plasma protein profiles by precentrifugation delay but not by repeated freeze-thaw cycles, as analyzed using multiplex proximity extension assays. Clinical Chemistry and Laboratory Medicine (2017).
2. Enroth S., et al., Effects of Long-Term Storage Time and Original Sampling Month on Biobank Plasma Protein Concentrations, EBioMedicine (2016).
3. Berglund M. et al., Protein Detection Using the Multiplexed Proximity Extension Assay (PEA) from Plasma and Vaginal Fluid Applied to the Indicating FTA Elute Micro Card™. Journal of Circulation Biomarkers (2016).
4. Björkesten et al., Stability of Proteins in Dried Blood Spot Biobanks. Molecular & Cellular Proteomics 16.7 (2017).
5. International Society for Biological and Environmental Repositories, ISBER Best Practices: Recommendations for Repositories Fourth Edition (2018).

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