White paper

PEA – a high-multiplex immunoassay technology with qPCR or NGS readout

Introduction

In the wake of genomics, the study of proteins is now emerging as the new frontier for understanding real-time human biology. Protein biomarker discovery enables identification of signatures with pathophysiological importance, bridging the gap between genomes and phenotypes. This type of data may have a profound impact on improving future healthcare, particularly with respect to precision medicine, but progress has been hampered by the lack of technologies that can provide reliable specificity, high throughput, good precision, and high sensitivity.

PEA technology – taking affinity-based protein measurements to a new level

The Proximity Extension Assay (PEA) technology (1) combines the best of antibody- and DNA-based methodologies to provide unique, enabling tools for protein biomarker discovery and development. The technique was commercialized by Olink Proteomics AB to develop its range of Olink® biomarker panels. PEA successfully merges an antibody-based immunoassay with the powerful properties of polymerase chain reaction (PCR), and readout using either quantitative real-time PCR (qPCR) or Next Generation Sequencing (NGS). This results in a scalable, multiplex and highly specific method where the concentration of hundreds of protein biomarkers can be quantified simultaneously.

The basis of PEA (Fig. 1) is a dual-recognition immunoassay, where two matched antibodies labelled with unique DNA oligonucleotides simultaneously bind to a target protein in solution. This brings the two antibodies into proximity, allowing their DNA oligonucleotides to hybridize, serving as template for a DNA polymerase-dependent extension step. This creates a double-stranded DNA "barcode" which is unique for the specific antigen and quantitatively proportional to the initial concentration of target protein. The hybridization and extension are immediately followed by PCR amplification. As will be described later, the resulting DNA amplicon can then be quantified either by microfluidic qPCR on a Fluidigm® Biomark instrument, or on the Illumina® NovaSeq platform, depending on the specific Olink product used.

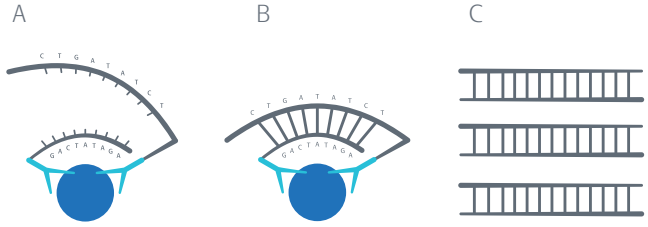

Figure 1. Main pre-readout steps in PEA. (A) Antibody pairs, labelled with DNA oligonucleotides, bind target antigen in solution. (B) Oligonucleotides that are brought into proximity hybridize and are extended by a DNA polymerase. (C) This newly created piece of DNA barcode is amplified by PCR ready for readout by NGS or qPCR.

Technical advantages of PEA

Traditional immunoassays do not lend themselves well to multiplexing since cross-reactive binding of antibodies contribute to the signal readout. This problem escalates exponentially with the degree of multiplexing. In contrast, the DNA-based readout of PEA circumvents this by requiring both dual recognition of correctly matched PEA probes (DNA-labeled antibodies), and DNA sequence-specific protein-to-DNA conversion to generate a signal. This provides a highly scalable method with an exceptional specificity (Fig. 2).

The exponential amplification properties of PCR are utilized in PEA to achieve a strong readout signal, providing assay sensitivity on par or better than traditional enzyme-linked immunosorbent assays (ELISAs). Importantly, this also means that extremely small sample volumes are needed to measure large numbers of proteins simultaneously, which is greatly beneficial when precious samples are in limited supply, such as in studies using human samples from clinical cohorts or biobank material. For the Olink qPCR readout

portfolio, this equates to 1 µL for each 96-plex panel, and for the NGS-based, Olink® Explore product, just 3 µL for 1536 assays. An additional benefit of the low sample volume is that concentrations of potentially interfering substances are minimized, which in conjunction with specifically tailored blocking reagents in the PEA protocol reduces sample matrix interference to a minimum.

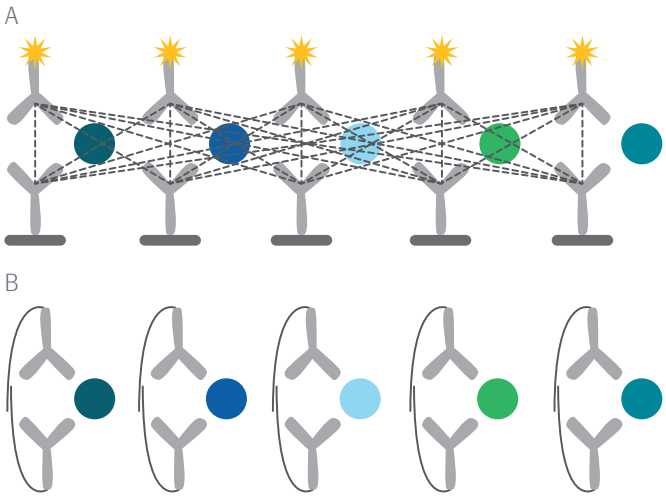

Figure 2. Overcoming the limitations of multiplexed immunoassays. (A) The problem of cross-reactive interactions when multiplexing a traditional immunoassay. (B) Cross-reactive interactions in PEA are not detected.

A unique and robust technology enabling two readout alternatives

The requirements for protein biomarker analysis can vary considerably among different applications, from hypothesis-driven, highly targeted investigations to broad studies aimed at screening large numbers of samples against many different proteins simultaneously. Over the course of several years, Olink developed an extensive range of 96-plex biomarker panels with qPCR readout, each focused around a specific disease area or biological process. This offers a uniquely flexible proteomics solution, enabling studies of anywhere from 96 to over 1100 proteins, depending on the selection of panels used. There is, however, a clear and growing demand for very large studies that can exploit the enormous potential knowledge held within the low abundant plasma proteome. Such studies require as many proteins as possible to be robustly measured in large numbers of samples. In response to these needs, Olink further developed its PEA technology to enable readout by NGS, enabling a substantial increase in throughput for a significantly expanded protein library, as well as providing a more cost-efficient solution.

The current Olink portfolio therefore includes products that use one of two main instrument platforms. User needs steer the choice of readout alternative, as will be described in the following sections.

PEA with NGS readout for large-scale protein biomarker discovery

Olink PEA technology with readout on Illumina's NovaSeq instrument enables a 384-plex format. Olink Explore 1536 is the first product on this platform aimed at large-scale plasma or serum sample studies. Typically, 1536 assays are measured for 88 customer samples plus 8 control samples per run. The 1536 assays are arranged in four 384-plex panels run in parallel, generating over 130 000 data points in less than 36 hours.

NGS in the Olink context

Conventional NGS is used to identify unknown DNA sequences that are mapped against a reference genome for applications such as rapid whole genome sequencing or mutation screening. NGS data is used for a different purpose in the Olink technology where known DNA sequences from PEA amplicons are counted. The matched NGS counts then represent the original protein concentration in the sample.

The molecular design of the amplicon incorporates a forward and a reverse DNA-barcode that contain assay ID information. A third DNA-barcode holds the sample ID information (Fig. 3). This makes it possible to detect 384 proteins in 96 samples simultaneously using only 0.8 uL plasma or serum sample.

Raw data in the analysis are matched counts of known sequences, where the forward and reverse barcodes are matched with a given index. Only counts with an exact sequence match are approved and included for further analysis. The barcode sequences differ significantly from each other and therefore the risk of counting sequences with erroneous bases is negligible. A semi-automated workflow with pipetting robots minimizes the number of manual pipetting steps, contributing to the exceptional repeatability and reproducibility obtained using the PEA technology.

An additional layer of precision is achieved through normalization using specifically engineered internal controls that are added to each sample and are utilized to reduce intra-assay variability (Fig. 4). These comprise one control for the inclubation, one for

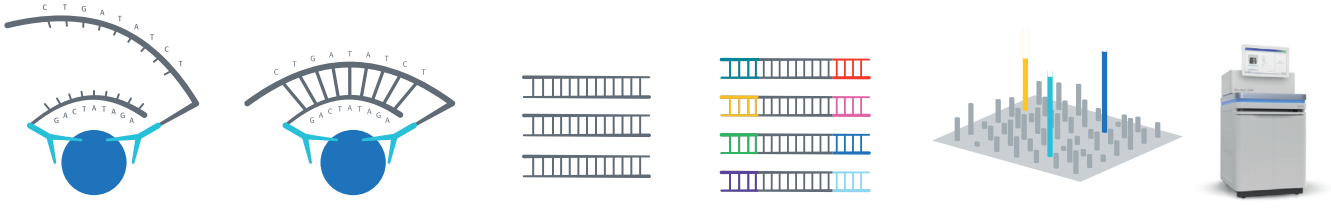

Figure 3. Barcoding of each protein and sample for NGS readout.

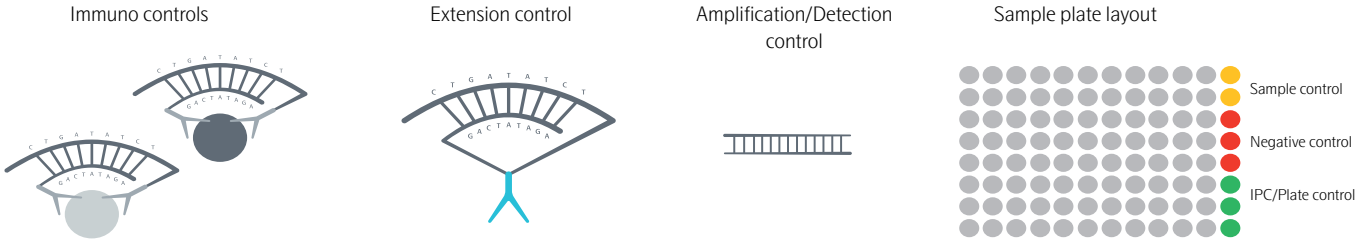

Figure 4. Internal controls for PEA.

the extension and one for the amplification step. Together, these internal controls monitor each step in the PEA protocol. Triplicates of an external negative control sample and a plate control sample are included in each sample plate and are used to improve inter-assay precision, allowing for optimal comparison of data derived from multiple runs.

Two external sample controls are included in the control strip to estimate the precision (CV). Finally, three biomarker proteins (IL-6, IL-8 and TNF-α) are measured independently in each of the four 384-plex panels, serving as an additional quality control.

PEA with qPCR readout for targeted protein biomarker discovery

PEA with qPCR readout on Fluidigm's Biomark instrument is generally carried out as a 96-plex assay. This well-established platform is available as a range of Olink® Target 96 panels, each quantifying 92 biomarkers focused around a disease area or biological process. The final detection of the 92 unique DNA barcodes in the PEA protocol is performed using microfluidic qPCR, enabling a high throughput. Typically, 88 customer samples and 8 control samples are assayed against 92 different proteins and the 4 internal control assays per run, generating over 8 000 data points in less than 24 hours. The use of automated microfluidics also reduces the number of manual pipetting steps needed, contributing to the exceptional repeatability and reproducibility obtained using the PEA technology (Fig. 5).

In line with the PEA protocol with NGS readout, data is normalized using specifically engineered internal controls that are added to each sample (Fig. 4). The normalization step helps reducing intra-assay variability. The controls for the qPCR method include two incubation controls and one each for the extension and detection steps. Together, these internal controls monitor each step in the PEA protocol. Triplicates of a negative control sample and triplicates of an inter-plate control (IPC) sample are included on each sample plate, and are used to improve precision between runs.

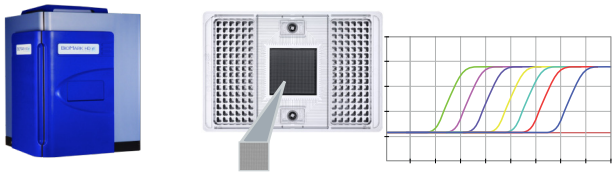

Figure 5. Readout of extension/amplification products by microfluidic qPCR.

While the assays included in the 96-plex panels have been most vigorously validated on plasma and serum samples, many have also been shown to work very well with an array of sample types, including cerebrospinal fluid (2), dried blood spots (3), and tissue lysates, such as atherosclerotic plaques (4), and tumor biopsies (5).

Summary

PEA is a method well suited for medium-to-large-scale protein biomarker studies, offering good sensitivity; rapid, high-throughput analysis; and exceptional specificity at high-multiplex levels. A portfolio of solutions for a broad range of applications from highly targeted, hypothesis-driven studies to the largest screening projects is available:

Olink Explore 1536 (NGS readout) is the ideal solution for scientists who wish to run high throughput studies with large numbers of human serum or plasma samples against the complete library of proteins.

The Olink Target 96 range of panels (qPCR readout) provides a high quality and flexible offering for more targeted investigations using one or several panels most relevant to the subject of study, or when sample matrices other than serum or plasma are measured.

Whichever platform is used, Olink technology enables scientists to search for new, actionable protein signatures within the low abundant plasma proteome that can be applied to liquid biopsy investigations to improve disease detection, aid more personalized healthcare, and allow a better understanding of real-time human biology.

References

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1129, v1.0, 2020-05-26