PEA: An enabling technology for high-multiplex protein biomarker discovery

In the wake of genomics, the study of proteins is now emerging as the new frontier for understanding complex biological systems. 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 high throughput, good precision, and high sensitivity.

Technology overview

The Proximity Extension Assay (PEA) is a molecular technique optimized to meet these demands (1) and 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 PCR and quantitative real-time PCR (qPCR), resulting in a multiplexable and highly specific method where up to 92 protein biomarkers can be quantified simultaneously.

The basis of PEA is a dual-recognition immunoassay, where two matched antibodies labelled with unique DNA oligonucleotides simultaneously bind to a target protein in solution (Fig 1A). This brings the two antibodies into proximity, allowing their DNA oligonucleotides to hybridize, serving as template for a DNA polymerase-dependent extension step (Fig 1B). 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 (Fig 1C), and the amplicon is then finally quantified by microfluidic qPCR (Fig 1D).

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 (Fig 2A). In contrast, the DNA-based readout of PEA circumvents this by requiring both dual recognition of correctly matched PEA probes, and DNA sequence-specific protein-to-DNA conversion to generate a signal (Fig 2B). This provides a highly scalable method with an exceptional readout specificity (Fig 2C).

The exponential amplification properties of PCR are utilized in PEA to achieve a strong readout signal, providing assay sensitivity on par with traditional enzyme-linked immunosorbent assays (ELISAs). Importantly, this also means that only 1 µL of sample is needed to measure 92 different 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. An additional benefit of the low sample

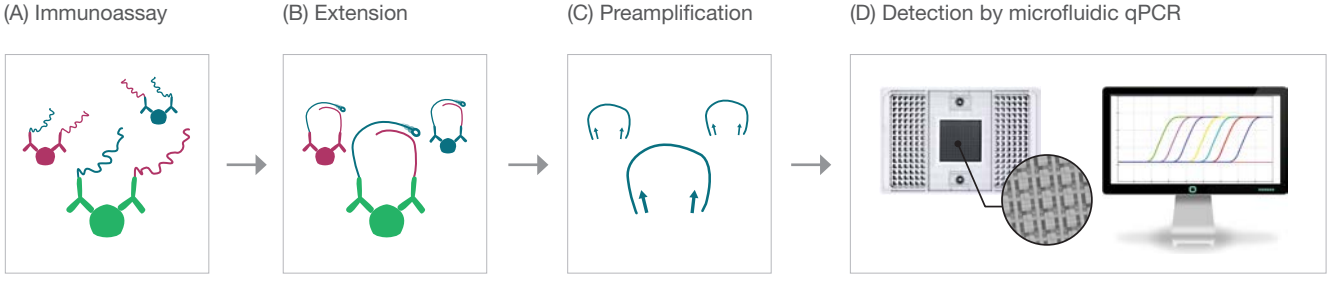
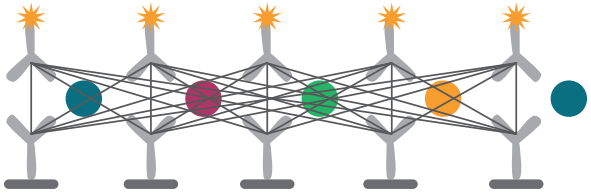


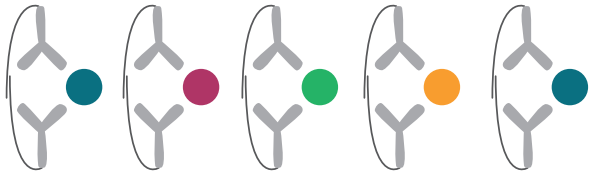
Fig 1. Overview of the PEA technology. (A) 92 Antibody pairs, labelled with DNA oligonucleotides, bind target antigen in solution. (B) Oligonucleotides that are brought into proximity hybridize, and are extended by a DNA polymerase. (C) This newly created piece of DNA barcode is amplified by PCR. (D) The amount of each DNA barcode is quantified by microfluidic qPCR.

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. While the assay has been most vigorously validated on plasma and serum samples it has 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).

(A) Traditional sandwich immunoassay



(B) PEA technology



(C) Scalability with PEA technology

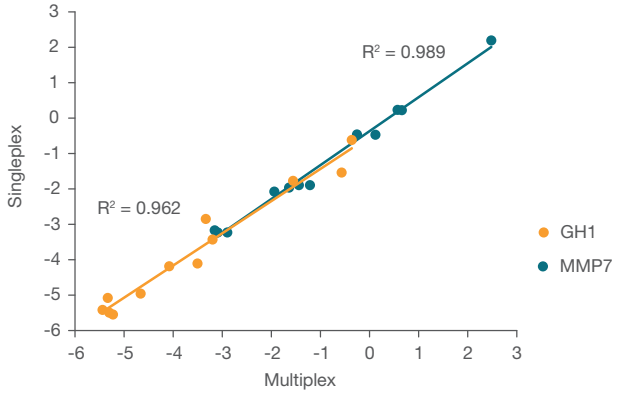


Fig 2. Scalability of PEA. (A) The problem of cross-reactive interactions when multiplexing a traditional immunoassay. (B) Cross-reactive interactions in PEA are not detected. (C) Correlation of two protein assays when run in PEA single-plex versus as part of a 92-plex PEA panel. Correlation data presented as dCq.

The final detection of the 92 unique DNA barcodes in the PEA protocol is performed using microfluidic qPCR, enabling a very high throughput. Typically, 90 samples are assayed against 92 different proteins per run, generating over 8000 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.

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. These controls include two for the immunoassay step and one each for the extension and detection steps. Together, these monitor each step in the complete PEA protocol (see Fig 1). An external interplate control sample is also included on each plate, and is used to improve interassay precision, allowing for optimal comparison of data derived from multiple runs.

Conclusions

In summary, PEA is a method well suited for large-scale studies of precision proteomics, offering good sensitivity; rapid, high-throughput analysis; and exceptional specificity at high-multiplex levels. Commercialized as a broad range of 92-plex Olink biomarker panels, it enables scientists to cast a wide net in the search for new protein signatures, which can be applied to improve disease detection, make available more personalized health care, and allow a better understanding of biology.

References

1. E. Assarsson *et al.*, Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. *PLOS ONE* **9**, e95192 (2014).
2. E. Bäckryd, L. Tanum, A. L. Lind, A. Larsson, T. Gordh, Evidence of both systemic inflammation and neuroinflammation in fibromyalgia patients, as assessed by a multiplex protein panel applied to the cerebrospinal fluid and to plasma. *J. Pain Res.* **10**, 515–525 (2017).
3. J. Björkesten *et al.*, Stability of proteins in dried blood spot biobanks. *Mol. Cell Proteomics* doi:10.1074/mcp. RA117.000015. [Epub ahead of print] (2017).
4. M. Wigren *et al.*, Decreased levels of stem cell factor in subjects with incident coronary events. *J. Intern. Med.* **279**, 180–191 (2016).
5. A. Loskog *et al.*, Immunostimulatory AdCD40L gene therapy combined with low-dose cyclophosphamide in metastatic melanoma patients. *Br. J. Cancer* **114**, 872–880 (2016).

1074, v1.0, 2017-12-07

Olink Proteomics
Dag Hammarskjölds v. 52B
SE-752 37 Uppsala, Sweden
www.olink.com