OLINK
BIOSCIENCE

Sensitive detection of cytokines in 1-µl serum samples using Proseek®

Olink Bioscience's Proseek protein assay enables sensitive detection and quantification of proteins in a 1-µl sample volume. With the Proseek Assay Development kit, a new assay can rapidly be developed for any target protein with appropriate antibodies available. Proseek's minimal sample consumption and excellent assay performance make it highly suitable for analyzing biomarkers and cytokines in precious biological samples.

Biomarker research in serological samples and in small laboratory animals is often limited by the large sample volume consumption that traditional technologies require.The Proseek Assay Development kit has been created to lower these hurdles. By selecting appropriate antibodies to combine with this kit, researchers can rapidly set up new target-specific assays consuming only 1 µl of sample per test, with excellent sensitivity. The Proseek reagents are based on Proximity Extension Assay technology, which uses a pair of DNA oligonucleotides linked to a set of matched antibodies to detect the target protein in a homogeneous assay[1]. Upon binding of the two probe reagents to the target proteins, a weak DNA duplex is formed, then is extended by a DNA polymerase to generate a real-time PCR amplicon. This assay, which has no washing steps, transforms the detection of the target protein into a DNA readout.

The Proseek Assay Development kit

In the first step, the user conjugates two matched monoclonal antibodies, or a single batch of antigen affinity–purified polyclonal antibody split into two fractions, to different oligo sequences, creating Proseek probes A and B. The Proseek probes are then incubated with the samples and a dilution series of the antigen standard, and the probes are allowed to bind pairwise to the target protein. This dual recognition ensures assay specificity. When two Proseek probes come into close proximity, a new PCR target sequence is formed by proximity-dependent DNA polymerization.The user then detects and quantifies the PCR sequences using standard real-time PCR (**Fig. 1**). Researchers can easily export the results file from their real-time PCR instrument to spreadsheet or curve-fitting software, where users may calculate average Ct, ΔCt and s.d., and convert these values to concentrations. The assay procedure does not include any washing steps, reducing the hands-on time and the possibility for human error.

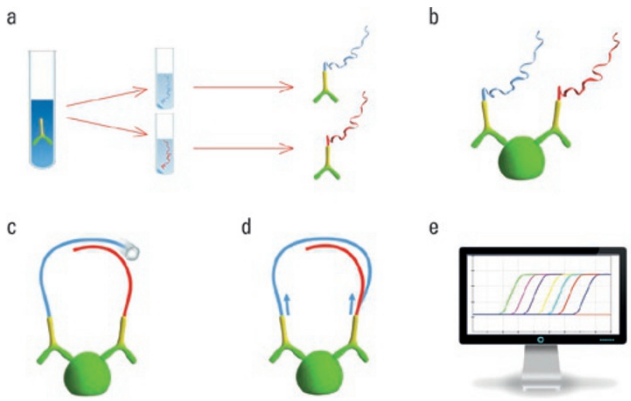

Figure 1 | Principle of the Proseek assay. (**a**) Target-specific antibodies are conjugated to different oligos (blue and red, respectively), creating Proseek probes A and B. (**b**) The Proseek probes are then incubated with the samples and a dilution series of the antigen standard in a 96-well plate or test tubes, allowing the probes to bind to the target protein. (**c**) Probes that are bound in close proximity are extended through a DNA-polymerization event, creating a real-time PCR amplicon. (**d**,**e**) The resulting extension products are combined with a real-time PCR mix (**d**), and amplification of the DNA occurs in a standard real-time PCR instrument (**e**).

Quantifying IL-8 levels in biobanked plasma from colorectal cancer patients

Interleukin 8 is an inflammatory cytokine previously reported to be elevated in serum and plasma of colorectal cancer (CRC) patients, and it may correlate to metastasis, as seen using standard immunoassay technologies[2]. Here we used the Proseek Assay Development kit to develop an IL-8 assay from a polyclonal anti–IL-8 antibody (AF-208-NA) and analyzed plasma samples from 16 CRC patients and 16 healthy controls for IL-8 levels (**Fig. 2**). The assay was performed according to

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the Proseek standard protocol, and a calibration curve generated from a dilution series of the IL-8 antigen (293-VE-010) was used to assess assay sensitivity and quantify protein concentrations in the samples. The sensitivity, defined as the lowest detectable concentration that significantly exceeds the background level, was calculated at 0.11 pM (0.88 pg ml^{-1}), equivalent to that of high-sensitivity chemiluminescent ELISAs. The colorectal cancer patients showed higher levels of IL-8 compared to the healthy controls ($P = 0.04$, Student's *t*-test), indicating a possible involvement of IL-8 in the metastasis of colorectal cancer. Examples of other assays that have been set up and evaluated for use in human serum and plasma samples measure CA-125, CA-242, CRP, EGFR, GDNF, IL-6, IL-17, IP-10, PSA and VEGF (see http://www.olink.com for details).

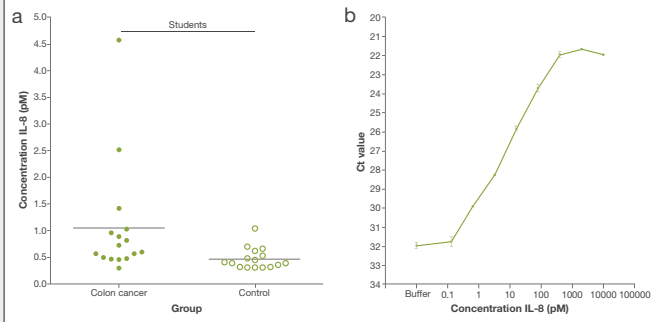
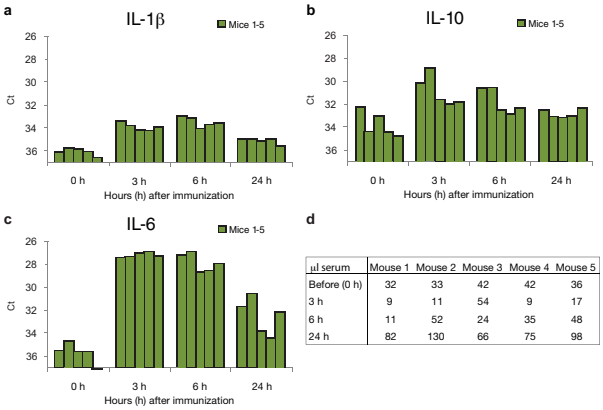


Figure 2 | Quantification of IL-8 levels in colon cancer and controls. (**a**) Measured IL-8 levels in colorectal cancer and control samples after conversion of Ct values to concentrations. (**b**) Calibration curve for IL-8, consisting of seven concentrations between 0.1 and 10,000 pM, and a zero buffer. Average Ct values of triplicates are plotted on the *y*-axis and concentrations on the *x*-axis. Error bars indicate s.d.

Monitoring cytokine levels in mice after immunization with lipopolysaccharide

IL-1β, IL-10 and IL-6 are typical inflammation cytokines, produced by the immune system in response to stimuli such as infection and pathogens. We rapidly set up assays for these markers using the Proseek Assay Development kit in combination with antibodies (AF-401-NA, AF-417-NA and AF-406-NA) to analyze cytokine expression levels in mice after immunization with the inflammatory agent lipopolysaccharide (LPS). Serum samples from five mice were collected before immunization (0 h) and 3, 6 and 24 h after immunization with LPS. For most of the mice and time points, less than 50 µl of serum was obtained from each mouse, making it difficult to analyze these samples for three different cytokines, and in duplicates, using conventional immunoassays (**Fig. 3d**). However, by taking advantage of Proseek's capability to consume only 1 µl of sample per reaction and its high assay performance, we were able to accurately monitor changes of IL-1β, IL-10 and IL-6 levels in these small sample volumes. The Proseek results showed significantly higher levels of all cytokines 3 h after as compared to before LPS immunization, followed by a gradual decrease in cytokine level with time (**Fig. 3**).



µl serum	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
Before (0 h)	32	33	42	42	36
3 h	9	11	54	9	17
6 h	11	52	24	35	48
24 h	82	130	66	75	98

Figure 3 | Cytokine detection in mice. (**a**–**c**) Cytokine detection in mouse samples before immunization (0 h) and 3, 6 and 24 h after immunization with LPS (**a**–**c**). Ct values from real-time PCR are plotted on the *y*-axes for IL-1β (**a**), IL-10 (**b**) and IL-6 (**c**). (**d**) Serum volumes obtained per mouse at each collection time point.

Conclusions

Because it consumes only minute amounts of sample, the Proseek assay is ideal for making better use of precious biological materials—for example, plasma or serum from limited biobanked material and small animal models. The low sample consumption makes it possible to detect multiple biomarkers, increase the number of replicates and generate more information from less sample volume compared to conventional immunoassays. Proseek also delivers excellent assay performance from a simple working procedure and a user-friendly protocol without washing steps. The technology, based on simple reagents, ensures specificity, sensitivity and reliability, enhancing confidence in the results. Furthermore, this assay system offers the benefits of detection using real-time PCR, allowing quantification of both absolute and relative protein levels.

1. Lundberg, M., Eriksson, A., Tran, B., Assarsson, E. & Fredriksson, S. Homogeneous antibody-based proximity extension assays provide sensitive and specific detection of low-abundant proteins in human blood. *Nucleic Acids Res.* doi:10.1093/nar/gkr424 (6 June 2011).
2. Ueda, T., Shimada, E. & Urakawa, T. *et al.* Serum levels of cytokines in patients with colorectal cancer: possible involvement of interleukin-6 and interleukin-8 in hematogenous metastasis. *J. Gastroenterol.* **29**, 423–429 (1994).