inducible siRNA expression systems may provide an interesting alternative in such cases[6]. For example, cells could be grown on a large scale before induction of the knockdown, which may be beneficial for proteomic analysis. Nonetheless, in targeting nonessential proteins, stable "knockdown" cells may be of great value when studying inducible processes such as UV or other irradiation damage response, host–pathogen interactions, or cell differentiation.

Considering all the pros and cons of expressed versus synthetic siRNAs, it is probably most effective to begin the search for highly effective siRNAs with synthetic, ready-to-use duplex RNAs of defined sequence and length, and to select the synthetic sequences such that they are readily compatible with the sequence requirements for expression within U6 or H1 RNA expression cassettes. This will first entail ensuring that in the U6 snRNA and H1 RNA systems that the +1 position is a guanosine[7,9] and an adenosine, respectively. In addition, it will require that uridines be present in the 3′-terminal position encoded by the oligothymidine Pol III terminator signal sequence[9].

Ultimately, the possibility of stable expression of siRNAs may pave the road for new gene therapy applications, such as treatment of persistent viral infections. Incorporation of siRNA expression cassettes into retroviral vectors may also allow the targeting of primary cells previously refractory to siRNA or plasmid DNA transfection[8]. Considering the high specificity of siRNAs[4–7], the approach should allow the targeting of disease-derived transcripts with point mutations, such as *RAS* or *TP53* oncogene transcripts, without alteration of the remaining wild-type allele. Finally, because of the automation developed for high-throughput sequence analysis of the various genomes, the DNA-based methodology may also be a cost-effective alternative for automated genome-wide loss-of-function phenotypic analysis, especially when combined with miniaturized array-based phenotypic screens[11].

1. Elbashir, S.M. *et al*. *Nature* **411**, 494–498 (2001).
2. Caplen, N.J. *et al*. *Proc. Natl. Acad. Sci. USA* **98**, 9742–9747 (2001).
3. Harborth, J. *et al*. *J. Cell Sci.* **114**, 4557–4565 (2001).
4. Brummelkamp, T.R. *et al*. *Science* 21 March 2002 (10.1126/science.10668999).
5. Lee, N.S. *et al*. *Nat. Biotechnol.* **20**, 500–505 (2002).
6. Miyagishi, M. & Taira, K. *Nat. Biotechnol.* **20**, 497–500 (2002).
7. Paul, C.P. *et al*. *Nat. Biotechnol.* **20**, 505–508 (2002).
8. Paddison, P.J. *et al*. *Genes & Dev.* **16**, in press (2002).
9. Paule, M.R. & White, R.J. *Nucleic Acids Res.* **28**, 1283–1298 (2000).
10. Myslinski, E. *et al*. *Nucleic Acids Res.* **29**, 2502–2509 (2001).
11. Ziauddin, J. & Sabatini, D.M. *Nature* **411**, 107–110 (2001).

Bringing picomolar protein detection into proximity

Combining target recognition by two aptamers, enzymatic ligation, and PCR, the proximity ligation method enables the detection of minute amounts of proteins.

Michael Famulok

Thousands of proteins with potential diagnostic and/or therapeutic applications are expected to emerge from the various genome projects. Proteins in different tissues or individual cells can be up- or downregulated in response to internal or external stimuli, signal transduction, transcriptional control, medication, disease, or pathogens. The ability to monitor slight differences in the amounts of proteins and other biomolecules within the smallest possible detection volumes down to the single-cell level is of utmost importance not only for proteomics research but also for diagnostic and technological purposes. In this issue, Fredriksson *et al.*[1] describe a new method, proximity ligation, that allows the detection and quantification of minute amounts of a specific protein.

Methods currently in widespread use for research requiring standard protein detection include two-dimensional gel electrophoresis, mass spectrometry, and antibody-based detection by sandwich enzyme-linked immunosorbent assays (ELISAs) or western blotting. Though sufficient for most standard applications, these techniques are not sensitive enough to meet the increasingly stringent detection limits required in the postgenomics era. For example, many proteins, such as cytokines and certain kinases, exert their functions at concentrations considerably lower than the thresholds of detection attainable by these methods. To overcome these limitations, variations of the sandwich ELISA with markedly increased sensitivity, such as the "immuno–polymerase chain reaction" (immuno-PCR)[2] and a technique termed "immunodetection amplified by T7 RNA polymerase" (IDAT)[3], have been developed. These methods combine antigen recognition by a biotin-tagged antibody with PCR amplification of a biotinylated reporter DNA, linking the two to one another via a streptavidin molecule. Tags other than biotin, alternative bridging moieties, or covalent coupling methods have also been used in immuno-PCR experiments. When performed under "real-time PCR" conditions, in which a fluorigenic oligonucleotide probe is used to measure PCR product accumulation, these methods can provide very accurate and highly sensitive quantification of the PCR products.

Fredrickson *et al.* do not use any antibodies, tags, or linker molecules and yet achieve levels of protein detection competitive with those of immuno-PCR. They have used a clever strategy whereby an "antigen" is detected by nucleic acid–based receptor molecules, so-called aptamers[4]. As a test case, the researchers use a pair of DNA aptamers that binds to the homodimer of the target analyte protein, platelet-derived growth factor B-chain (PDGF-BB). Each aptamer has a different DNA-sequence extension that does not interfere with its folding and is not required for target recognition. Binding of the aptamer pair brings the ends of the oligonucleotide extensions into close spatial proximity (Fig. 1A) so that a "splint" oligonucleotide can hybridize to both ends, which are subsequently ligated together by T4 DNA ligase. The ligated species can then act as a PCR template and the amplified PCR product can be monitored and quantified under real-time PCR conditions, whereas no signal is obtained with unligated probes (Fig. 1B).

The sensitivity that can be achieved with the method is remarkable. As few as 24,000 molecules, or 4×10^{-20} moles, of the PDGF-BB protein could be detected, approximately 1,000-fold fewer than could be

Michael Famulok is a professor at the Kekulé Institute of Organic Chemistry and Biochemistry, Universität Bonn, Gerhard-Domagk-Strasse 1, 53121 Bonn, Germany (m.famulok@uni-bonn.de).

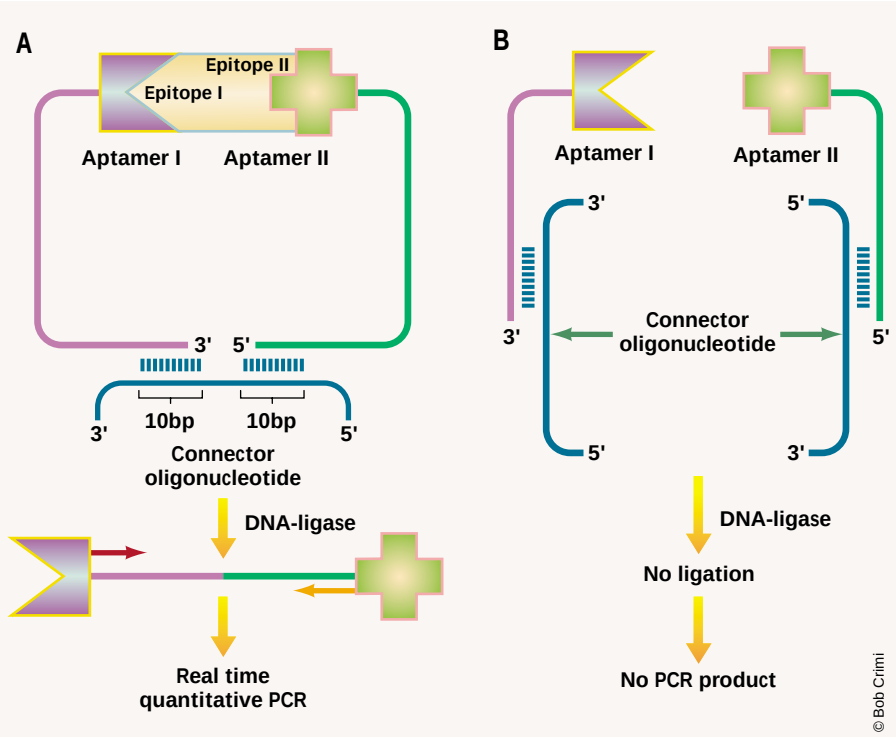

Figure 1. The proximity ligation assay can be 1,000-fold more sensitive than a conventional ELISA. (A) A pair of aptamers that bind either to distinct epitopes of the target protein or to the same epitope in a homodimer bring the free ends of their sequence extensions into close spatial proximity. This allows the core sequence of the connector oligonucleotide to hybridize to both ends, acting as a template for enzymatic ligation. Subsequently, ligated sequences can be PCR amplified using specific primer sequences (brown and orange arrows). Detection of the PCR product is achieved under conditions of real-time quantitative PCR. (B) Without the target protein, the connector oligonucleotide cannot act as a ligation template.

detected in a standard sandwich ELISA assay for the same target. Although the detection sensitivity is in the same ballpark as for immuno-PCR, a major advance of the proximity ligation assay as compared with immuno-PCR is that the whole assay can be performed in a homogenous format that does not include a single wash or separation step. This saves considerable time and should make the method suitable for automation. The assay also has potential for application in clinical laboratories or hospitals, as it can detect PDGF-BB present in crude biological samples such as fetal calf serum, cerebrospinal fluid, cell culture media, and human blood serum[1].

Like immuno-PCR, proximity ligation is prone to certain restrictions connected with the nonlinear amplification ability of PCR that may limit the approach for quantification. Rolling-circle PCR (ref. 5) or IDAT (ref. 3) may easily overcome these restrictions. A potentially more serious limitation, however, could be the difficulty of generalizing the assay. Fredriksson *et al*. obtained their most impressive result with a multimerized protein, the PDGF homodimer. To detect PDGF they used two aptamer-based proximity probes that recognize the same site on each dimer. In their second example, detection of a monomeric protein, the human blood clotting factor α-thrombin, required a pair of two different aptamers that recognize distinct sites on the protein. This example was selected for good reason: except for α-thrombin, there is not a single additional protein target for which a set of aptamers that bind different sites on the same protein has been isolated. Although in principle it should be possible to obtain aptamers that bind to distinct sites on a target, it is unclear at present whether this can be done with the same ease as for antibodies, and, more importantly, whether very many targets exist for which such different aptamers are selectable.

However, it is now well established that aptamers can form high-affinity binding sites for almost any given target protein, with dissociation constants as low as subpicomolar[6] and binding specificities that can be as good as, or sometimes even better than, those of antibodies[7]. Furthermore, aptamers have the added advantage of stability because chemical modifications, such as 2′-fluoro-2′-deoxypyrimidine residues, can render them nearly resistant against nuclease degradation[6]. Aptamer researchers will probably now look much more thoroughly at whether their selected libraries contain aptamer pairs that bind different target sites. Furthermore, with automated selection protocols available[8], the isolation of the required aptamer pairs may become an easier task than in pre-automation days because *in vitro* selections can focus on selecting high-affinity aptamers that bind more than one site on a target. In addition, there is no reason that the approach should be limited to aptameric receptors. Proximity ligation will likely work for antibody probes that are linked to a DNA sequence, similar to immuno-PCR, and it will be interesting to see what level of sensitivity can be achieved with "immuno–proximity ligation".

The extremely sensitive detection of molecules that can be achieved with these methods raises the question of whether it will be possible, by clever technical refinement and by transformation into array formats, to build proximity ligation–based sensors that may reach the capabilities of, for example, the sensor array of the mammalian olfactory epithelium. This may seem far-fetched, but in the future it should be possible to use these or similar technologies for the identification of many different individual "odors" (i.e., minimal alterations in the composition of different sets of proteins from various tissues or cells). Unlike the mammalian olfactory system, however, sensor arrays based on the proximity ligation method may not be of use in quantifying small molecules. The fact that two different epitopes will have to be bound, regardless whether antibodies or aptamers are used, would make it difficult to adapt the method to small organic molecules or even small peptides. Even so, using the approach in combination with other methodologies, such as allosteric ribozymes[9], may help to achieve this goal. Wherever its future lies, the proximity ligation method deserves a close look, as it represents a significant advance as compared with current methods for protein quantification.

1. Fredriksson, S. *et al. Nat. Biotechnol.* **20**, 473–477 (2002).
2. Sano, T., Smith, C.L. & Cantor, C.L. *Science* **258**, 120–122 (1992).
3. Zhang, H.-T., Kacharmina, J.E., Miyashiro, K., Greene, M.I. & Eberwine, J. *Proc. Natl. Acad. Sci. USA* **98**, 5497–5502 (2001).
4. Wilson, D.S. & Szostak, J.W. *Annu. Rev. Biochem.* **68**, 611–647 (1999).
5. Schweitzer, B. *et al. Proc. Natl. Acad. Sci. USA* **97**, 10113–10119 (2000).
6. Pagratis, N.C. *et al. Nat. Biotechnol.* **15**, 68–73 (1997).
7. Gold, L. *Harvey Lect.* **91**, 47–57 (1995).
8. Levy, M. & Ellington, A.D. *Bioorg. Med. Chem.* **9**, 2581–2587 (2001).
9. Breaker, R.R. *Curr. Opin. Biotechnol.* **13**, 31–39 (2002).