Development of an Immuno-PCR Assay
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WHAT IS IMMUNO-PCR?

The Polymerase Chain Reaction (PCR), developed in 1983, is a procedure that is used to amplify segments of DNA. It relies on a thermostable DNA polymerase enzyme for the synthesis of a new DNA strand which is complementary to the template strand. The DNA polymerase enzyme requires double-stranded starting points in order to synthesize a complementary sequence of bases to a single strand of DNA; these are provided as primers. Cyclical changes in temperature are used to control the activity of the DNA polymerase and the binding of the primers, and enable billions of copies of the sequence of interest to be created in a relatively short period of time. The amplified DNA can then be run out and visualized on a gel at the end-point of the PCR reaction.

During real-time PCR (rtPCR), which evolved in the early nineties, DNA amplification is monitored by a machine rather than relying on a gel for detection. Various techniques allow the PCR reaction to be followed, all of which link the amplification of DNA to the production of a fluorescent signal; this fluorescent signal is detected by a camera during each PCR cycle. As the number of DNA copies increases during the reaction, so does the level of fluorescence. rtPCR allows DNA amplification to be detected during the early phases of the reaction, and the major advantage of rtPCR over the traditional approach is that it can be used for quantitative analysis.

Immuno-PCR was first developed in 1992, and combines PCR with an enzyme-linked immunosorbent assay or ELISA (Sano et al, Immuno-PCR: Very Sensitive Antigen Detection by Means of Specific Antibody-DNA Conjugates, Science 258 (5079) 120–122). The coupling of antibody specificity to DNA amplification ensures that immuno-PCR is significantly more sensitive than an ELISA, however the technique has not been as extensively used as one might expect mainly due to the complexity of conjugating antibodies to oligonucleotides. Our Thunder-Link® technology enables antibody: oligonucleotide conjugation to be carried out in just a few simple steps, and will be discussed in more detail shortly.

During a conventional sandwich ELISA an antibody is bound to a microplate and used to capture an antigen in solution; addition of a second antibody that is also specific to the antigen enables the antigen to be quantified, provided relevant standards have been included in the assay. In the seminal experiments which were performed by Sano et al, Bovine Serum Albumin (BSA) was immobilized on to microtitre plates, and was then detected with a monoclonal antibody to which a streptavidin-protein A chimera was subsequently attached. Biotinylated linear DNA was then added to the wells, amplified by PCR and analyzed by agarose gel electrophoresis. This technique has subsequently evolved to incorporate rtPCR as a detection method.

DEVELOPMENT OF AN IMMUNO-PCR ASSAY TO QUANTIFY PROSTATE SPECIFIC ANTGEN (PSA)

In their 2005 publication, Lind and Kubista describe the development of an immuno-PCR assay to quantify PSA in patient serum samples (Lind et al, Development and evaluation of three real-time immuno-PCR assemblages for quantification of PSA, Journal of Immunological Methods 304 (2005) 107-116). PSA is a glycoprotein which is produced by the prostate gland and is a well-known marker for prostate cancer; PSA levels in the blood are usually extremely low, however elevated serum concentrations may be indicative of prostate disease.
The main steps of an immuno-PCR assay are as follows:

1. Immobilization of antibodies specific for the protein target to the surface of a vessel
2. Washing to remove unbound antibody
3. Addition of sample
4. Washing to remove unbound sample
5. Addition of a second specific antibody, coupled to a DNA molecule
6. Washing to remove unbound antibody
7. DNA amplification and detection

Lind and Kubista purchased two different monoclonal antibodies to PSA, and used one of these for microplate coating, while conjugating the other to a 67-base strand of linear DNA:

(5'-TGCCCTGCGTTATCTGCTCCATGTCGCAAGCCTCATAGTTAGGAACATTACATTGACGCAGG-3').

Preparation of the antibody-DNA conjugate is described in full detail within the publication; in summary this involved activation of the antibody with thiol groups and activation of the DNA through the addition of an N-terminal amine group, column-based purification and elution of the activated antibody and DNA, mixing of the two solutions, column-based purification and elution of the conjugate, and removal of free antibody and DNA.

Following production of the antibody-DNA conjugate, the immuno-PCR assay was developed and optimized. An overnight incubation was used to immobilize the capture antibody onto a microplate, after which the wells were washed and then blocked. During the blocking step, PSA standards or test serum samples were incubated with the antibody-DNA conjugate; these samples were then added to the microplate and incubated. After incubation the microplate wells were washed thoroughly. Two primers (5'-CCCTGCGTTATCTGCTCC-3' and 5'-CCTGCGTCAATGTAATGTTC-3') were used to generate a 65 base pair long product, which was fluorescently quantified by rtPCR.

To enable a comparison of the immuno-PCR assay with a sandwich ELISA, Lind and Kubista carried out an ELISA based on the same antibodies, with the detection antibody conjugated to horseradish peroxidase (HRP). 30 serum samples were analyzed in both assay formats; the correlation between the two assays was excellent, however the sensitivity of the immuno-PCR assay was found to be over 100 times higher than that of the ELISA (see Figure 2).
THE BENEFITS OF REAL-TIME IMMUNO-PCR

While traditional PCR is a rapid and relatively simple technique that is used to amplify DNA, rtPCR is currently regarded as the gold standard in the quantitative analysis of nucleic acids. Real-time immuno-PCR combines the exponential signal amplification of rtPCR with the specificity of an ELISA.

Although ELISA assays are highly sensitive due to the use of an enzyme as the reporting group, immuno-PCR provides greatly increased sensitivity and delivers a significantly larger quantification range. Immuno-PCR can be used to detect extremely low levels of the antigen, often at 1000-fold lower concentration than the levels which would be detected by an ELISA developed against the same target.

As well as enhanced sensitivity further benefits of immuno-PCR are that it requires no additional equipment to that which is used for rtPCR, it involves fewer incubation steps than an ELISA making it more robust and giving higher reproducibility, it is compatible with complex samples such as serum, and multiplexing is possible. Compared to many assay formats, immuno-PCR is also very affordable.

HOW CAN THUNDER-LINK® PLUS BE USED TO SIMPLIFY ANTIBODY-OLIGO BIOCONJUGATION?

Despite demonstrating significantly greater sensitivity and a much wider dynamic range than an ELISA, immuno-PCR is not yet the number one go-to immunodiagnostic assay. The main reason for this is that it is notoriously difficult and time-consuming to conjugate oligonucleotides to antibodies; furthermore the conjugation process can use considerable amounts of antibody, with losses being incurred during column-based purifications.

The Thunder-Link® PLUS technology which we have developed at Expedeon enables the conjugation of an antibody to an oligonucleotide in just a few simple steps. The conjugation process is unidirectional and allows only antibody-oligonucleotide complexes to be formed (antibody-antibody or oligonucleotide-oligonucleotide complexes are not produced). A positive control is included to demonstrate successful conjugation, and an optional purification step enables any unconjugated oligonucleotides to be removed. A brief overview of the Thunder-Link® PLUS conjugation protocol is as follows:

- Add the oligo to the oligo activation reagent vial
- Add the antibody to the antibody activation reagent vial
- Incubate both vials for 30 minutes at room temperature
- Wash the two desalt columns provided in the kit
- Add the activated oligo mix to the top of one column, push the liquid to the base of the column, and elute
- Add the activated antibody mix to the top of the other column, push the liquid to the base of the column, and elute
- Add the activated antibody and oligo together and incubate for 1 hour at room temperature
- Purify the antibody-oligo conjugate with our easy to use Conjugate Clean Up Reagent

Antibody-oligonucleotide conjugates are the next generation of tools in biomarker detection. They have huge potential for use in multiplexed protein diagnostic assays since different reporter oligonucleotides can be conjugated to antibodies against specific protein targets. Our Thunder-Link® PLUS oligo conjugation system enables antibody-oligo conjugates to be generated easily and efficiently, hugely increasing the accessibility of immuno-PCR across the scientific community. In addition to immuno-PCR, antibody-oligonucleotide conjugates are also applicable to other assay formats, including proximity ligation assays, proximity extension assays, and electrochemical proximity assays.

In addition to our easy-to-use Thunder-Link® PLUS kits, Expedeon also offers a range of custom services, including antibody/oligonucleotide micro-optimization and an antibody-oligonucleotide conjugation service.
MICRO-OPTIMIZATION SERVICE

Our unique conjugate micro-optimization services provide a fast and efficient way to optimize your conjugates using very small quantities of your antibody or protein, enabling you to find the best performing conjugate for your assay. Our experienced conjugation scientists use a combination of conjugation techniques, many of which are unique to Expedeon, to generate a range of different conjugates for you to test in your chosen application and up-scale for future requirements.

Further details of this service can be found on our website.