Application Note

High Sensitivity Detection of Antigens using Immuno PCR

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Key Words
Thermo Scientific™ Nunc™ MicroWell™, Thermo Scientific™ Nunc™ TopYield™, PCR ELISA, Immuno PCR, Antigens, nucleic acids.

Goal

The goal of this application note is to describe a sandwich Immuno PCR assay and the analysis of the Immuno PCR amplicons in a TopYield module plate. Further to use a standardized and reliable protocol for reagent preparation and analysis.

The detection of nucleic acids can be achieved at levels of a few molecules using the polymerase chain reaction (PCR). In contrast, the analysis of proteins using conventional methods, such as enzyme-linked immunosorbent assay (ELISA), hardly surpasses sensitivity levels below 1 x 10^{-18} mol of the antigen. By combining the enormous amplification power of PCR with antibody-based immuno-assays, Immuno PCR (I-PCR) allows the detection of proteins at a level of a few hundred molecules[1]. In this Application Note, a sandwich I-PCR (Fig. 1)[2] and the analysis of the I-PCR amplicons in a Nunc MicroWell format are described.

The successful establishment of I-PCR as a routine method is dependent on using a reaction vessel in a Nunc MicroWell plate format, with high protein binding capacity and sufficient thermal stability to allow both initial immuno-reactions and subsequent thermal cycling.

A standardised protocol for reagent preparation and a rapid and reliable method for the analysis and quantification of the amplified DNA are also required.

Methods

Preparation of Nunc MicroWell plates antibody coated plates are prepared using heat stable Thermo Scientific Nunc TopYield Strips. Add 30 µL of a capture antibody diluted at 20 µg/mL to each well.

Incubate overnight at 4°C. Wash three times with Buffer B (10 mm Tris, pH 7.3, containing 150 mm NaCl) and block overnight at 4°C with 150 µL of Buffer C (Buffer B containing 4.5% skim milk powder, 0.2% NaN_3, 5 mm EDTA and 1 mg/mL hering sperm DNA). Wash four times with Buffer D (Buffer B containing 0.05% Tween 20 and 5 mm EDTA). Streptavidin coated plates are used for the enzyme-based detection of I-PCR amplicons.

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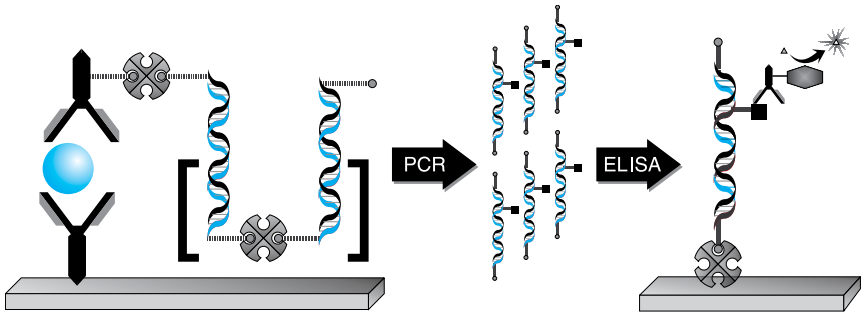

Fig. 1.

Immuno PCR detection of an antigen with subsequent quantitative analysis of product yields by PCR ELISA. Capture antibodies are immobilized on MicroWell plates to bind the antigen selectively. Sequential coupling of a biotinylated detection antibody, streptavidin, and a biotinylated DNA marker assemble a signal generating immuno-complex. Signal amplification by PCR using a biotinylated primer and a digoxigenin labeled nucleotide generates doubly labeled amplificate which can be quantified in a PCR ELISA. The PCR products are immobilized on streptavidin coated plates and analysed by anti-digoxigenin IgG-alkaline phospatase conjugate with either chromogenic or fluorogenic substrates.

Preparation of DNA Streptavidin Reagents

A bis-biotinylated DNA reporter fragment is prepared from M13mp18 DNA template by PCR using biotinylated primers. Conjugates of recombinant STV and the bis-biotinylated DNA fragment are prepared by adding 2 pmol of STV in 1 µL Buffer E (10 mm Tris-HCl, pH 7.3, 5 mm EDTA) to 13 µL of Buffer E, and subsequently, 1 pmol of the bis-biotinylated DNA in 1 µL Buffer E. The mixture is incubated for 15 minutes at room temperature.

Immuno PCR

The antigen-capture step of the sandwich I-PCR is carried out with 1:3-1:10 serial dilutions of the antigen containing sample. 30 µL of the diluted samples are added to the wells of the antibody coated Nunc TopYield Strips and incubated for one hour at room temperature. Wash three times with Buffer D. Add 30 µL of the biotinylated detection antibody (3.5 mg/mL in Buffer F). Add 30 µL oligomeric DNA streptavidin (5 pm in Buffer F). Incubate for 30 minutes. Wash six times with Buffer D and twice with Buffer B. PCR is carried out directly in the TopYield Strips in 29 cycles. PCR reaction mix contains 11-digoxigenin-dUTP to allow quantification of the I-PCR amplicons using the Nunc MicroWell assay described below.

Analysis of I-PCR Amplicons

I-PCR amplicons can be detected by gel-electrophoresis, or in a Nunc MicroWell plate format. The latter is advantageous, since it allows for quantification of a large number of samples, and also further enhances the sensitivity of I-PCR. I-PCR products are diluted 1:80 for colorimetric detection, or 1:800 for fluorescence detection with Buffer D, and 50 µL are added to the streptavidin coated Nunc MicroWell plate. Incubate for 45 minutes at room temperature. Wash five times with Buffer D. Add 50 µL of a 1:5000 dilution of anti-Digoxigenin IgG-alkaline phosphatase conjugate in Buffer D. Incubate for 45 minutes at room temperature. Wash three times with Buffer D and three times with Buffer B.

For colorimetric detection, add 200 µL of 1 M diethanolamine, pH 9.8, and 0.5 mm $MgCl_2$ containing 10 mm p-nitrophenyl phosphate. The reaction is quenched after 60 minutes at 37°C by adding EDTA. Read the result at 405 nm.

For fluorescence detection, AttoPhos substrate may be used, prepared according to manufacturer's instructions. The plates are incubated 30 minutes at 37°C and analysed on a fluorescence reader with 440 nm excitation and 550 nm emission wavelengths.

Results

A comparative study with a mouse IgG as model antigen using either a sandwich ELISA or sandwich I-PCR is shown in Fig. 2. As can be seen a 1:1000 enhancement in sensitivity is obtained from the employment of I-PCR instead of the ELISA technique.

The signal intensities of I-PCR are linearly dependent on the amount of antigen, and the dynamic range of the assay often exceeds five orders of magnitude.

The influence of the Nunc MicroWell plate used in the I-PCR is also evident from the curves in Fig. 2. TopYield Strips are compared with two other temperature resistant reaction vessels. Brand X, made of polyethylene, was coated with capture antibody as described above. Brand Y, containing a chemically activated surface capable of binding antibodies covalently as well as other proteins, was coated with capture antibody according to manufacturer's instructions. A sandwich- I-PCR was carried out for the detection of the mouse IgG antigen. Using TopYield, a sensitivity of less than 0.1 amol of antigen is seen in the I-PCR while the protein binding capacity of Brand X is not sufficient for an immuno-assay. The use of Brand Y plates leads to a limited sensitivity of about 100 amol of antigen.

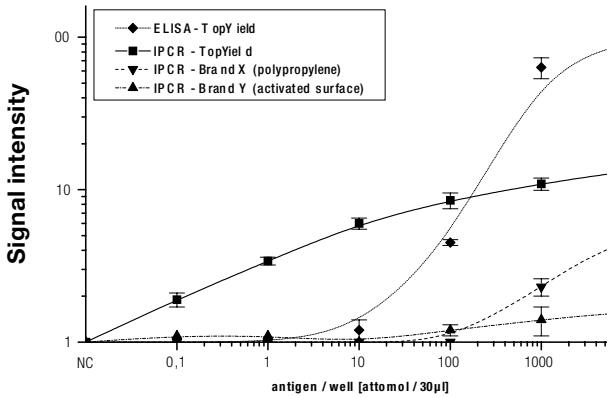

Fig. 2.

Detection of IgG using a direct immuno PCR assay with a subsequent AttoPhos PCR ELISA.

Detection of mouse and rabbit IgG was carried out from 1:10 serial dilutions of antigen in the range of 150 pg (1 fmol) to 1.5 fg (0.01 amol). The error bars indicate the maximum deviation observed for independent immuno PCR detections of the antigens.

References

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