Application Note

Thermo Scientific Nunc Immobilizer Streptavidin

Application example: PCR ELISA

Key Words

Immobilizer Streptavidin, coated plates, biotin-binding, PCR ELISA, low detection limit, low non-specific background.

Goal

The goal of this application note is to describe an easy and fast method for coupling of biotinylated oligonucleotides to Thermo Scientific™ Nunc™ Immobilizer™ Streptavidin plates. Further, to show a low detection limit and low non-specific background using PCR ELISA.

Nunc Immobilizer Streptavidin plates are manufactured using a patented photochemical method[1] for covalent coupling of ligands to polymer surfaces. Streptavidin, a high affinity biotin-binding protein isolated from Streptomyces avidinii, is covalently coupled via a polyethylene glycol spacer arm to the plate. The density, length and chemical composition of the spacer arms has been optimized to create a superior affinity surface. High signals, low non-specific binding and convenient room temperature storage are some of the benefits realized.

This note describes an easy and fast method for coupling biotinylated oligonucleotides to Nunc Immobilizer Streptavidin plates. We have demonstrated that the immobilized oligonucleotides are able to further hybridize in a specific manner with Digoxigenin (DIG) labeled PCR products.

Introduction

PCR ELISA is a very sensitive analytical technique that utilizes nucleic acid hybridization and immunoassay methodology [2,3,4]. Streptavidin-coated wells facilitate the application by making it easy to prepare the requisite solid phase "capture probe" surface.

Briefly, one first amplifies a target DNA via PCR in which a hapten (e.g. digoxigenin) containing nucleotide is included in the reaction mixture. This produces amplified DNA products that are labeled with the hapten. The hapten labeled DNA is then denatured and hybridized to a capture oligo that has been immobilized on an appropriate surface such as the surface of a microplate. A streptavidin coated plate to which the biotinylated oligo has been bound is a convenient and proven solid phase for this. After washing, the hybridized hapten labeled DNA is detected using a labeled (enzyme) anti-hapten antibody. The amount of bound labeled antibody is directly proportional to the amount of target DNA present in the original sample.

The example given demonstrates how the Immobilizer Streptavidin plate can be used to carry out this type of assay. In this model system, we show how target DNA, plasmid pUC 19 DNA[5], can be detected. The assay utilizes a streptavidin plate to which biotin labeled capture probe has been immobilized. The capture probe is a biotinylated oligo that is complementary to pUC. Target DNA (pUC DNA) is amplified via PCR using a digoxigenin containing nucleotide in the reaction mixture. The digoxigenin nucleotide is incorporated and the resulting hapten labeled PCR products are subsequently denatured and hybridized to the solid phase capture probe. Finally the bound hapten labeled amplified DNA is detected using HRP labeled anti-digoxigenin.

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Materials

- Immobilizer Streptavidin LockWell™ module plate, clear
- 5 x SSCT, pH 7.0 (5 x SSC (750 mm NaCl, and 75 mm Sodium Citrate) containing 0.05% (v/v) Tween 20)
- 2 x SSCT, pH 7.0 (2 x SSC (300 mm NaCl, and 30 mm Sodium Citrate) containing 0.05% (v/v) Tween 20)
- PBST, pH 7.2 (Phosphate Buffered Saline containing 0.05% (v/v) Tween 20)
- Hybridization solution (50 mm Sodium-Phosphate buffer pH 7.0)
- Denaturation solution (125 mm NaOH)
- Biotinylated capture probe
- 3.3´, 5.5´ tetramethylbenzidine "TMB one" Ready to Use
- Sulphuric acid 0.5 M

Procedure for coupling the biotinylated capture probe

1. Pre-wash the plate with 3 x 300 µL/well 5 x SSCT buffer. This is done to ensure improved sensitivity and high precision.
2. Prepare a solution of biotinylated capture probe in 5 x SSCT. We recommend a pre-optimization of the biotinylated capture probe concentration over the range of 0.01 to 0.5 µm.
3. Add the capture probe to the Immobilizer Streptavidin microplate/strip (100 µL/well).
4. Incubate the plate/strip with gentle agitation at room temperature for ≥30 min.
5. Aspirate the wells and wash with 2 x SSCT (3 x 300 µL/well).
6. The Immobilizer Streptavidin surface is now ready for the amplified target DNA sample.

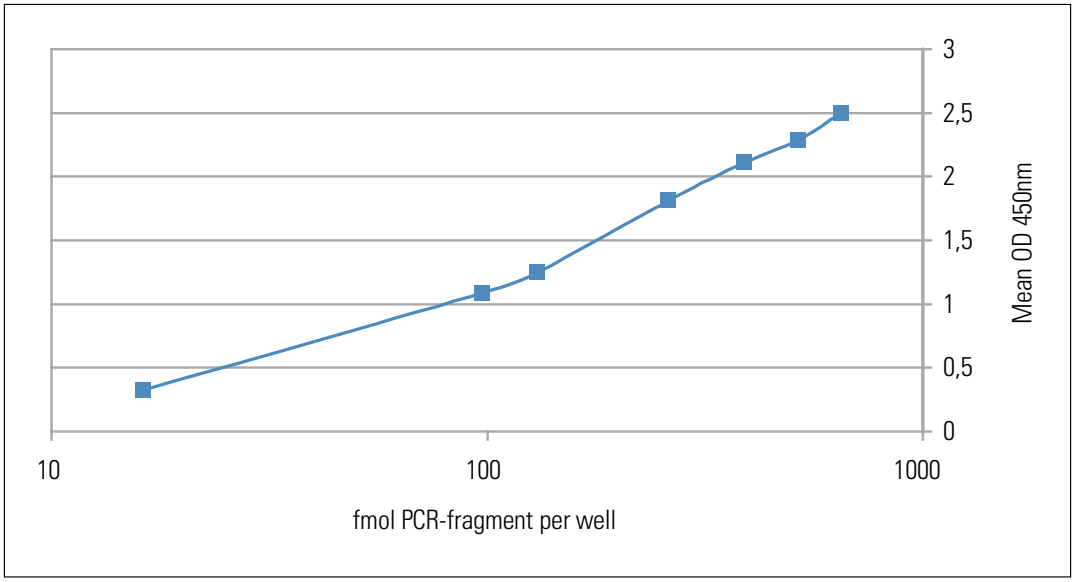
Amplification and denaturation of target

1. pUC DNA was PCR amplified using the following primers: 5´ - AAC AGC TAT GAC CAT-3´ and 5´ - GTA AAA CGA CGG CCA GT 3´ [6]. The PCR labeling kit was used following the manufacturers instructions. The target DNA was amplified using the following program: 7 min. at 94°C; 35 cycles (94°C 1min.; 45°C 1 min.; 72°C 1 min.); and final elongation 72°C 10 min. The yield was estimated by agarose gel electrophoresis.
2. The PCR products were denatured as follows; 5 µL, 4 µL, 3 µL, 2 µL, 1 µL, 0.75 µL and 0.14 µL of PCR products were added to Thermo Scientific Nunc MiniSorp tubes and incubated with 10 µL of denaturation solution. The solutions were incubated for 5 min. and then 100 µL of hybridization solution was added. Each solution was mixed and 100 µL was transferred to each well of the capture probe coated streptavidin strip plate (see step 6 above).

Hybridization and immunological detection

1. The hybridization solution (see step 2 above) was allowed to incubate in the wells for 60 min. at 37°C with gentle agitation.
2. The strips were aspirated and washed three times with 2 x SSCT buffer which was preheated to +37°C.
3. HRP-anti DIG, Fab fragment diluted 1:1000 in PBST were added to the strips (100µL/well). Note: This dilution was prepared in low-protein binding tubes.
4. The strips were incubated with gentle agitation at room temperature for 30 min.
5. The strips were aspirated and washed three times with PBST solution (3 x 300 µL/well).
6. TMB solution was added to the strips (100 µL/well) and incubated, in the dark, at room temperature for 10 min.
7. The enzyme reaction was stopped with H^2SO^4, 0.5 M (100 µL/well). The absorbance was measured at 450 nm using an ELISA reader.

Fig. 1



Summary

The results show that the Immobilizer Streptavidin plate is an excellent solid phase for carrying out a PCR ELISA test. Using this unoptimized assay, the magnitude of the signal is seen to be directly proportional to the amount of PCR fragment added to the well (see Fig. 1). Less than 100 fmol ($1x10^{-13}$ mol) per well could be easily detected. A low non-specific binding signal of 0.05 OD unit was observed in the assay even though no blocking procedure was used. Although PCR ELISA utilizes an antibody for signal generation, assays that employ directly labeled probes can be similarly performed.

Specifications

- Streptavidin coated area ~100 µL per well 96 well clear plate
- Total binding capacity for free biotin 5 ng/well (20 pmol/well)*
- Stable at room temperature for 18 months after manufacturing
- Coefficient of variation (CV) < 5% from well to well

*The binding capacity may vary depending on the size and steric properties of the biotinylated biomolecule being used.

References

1) Koch T, Jacobsen N, Fensholdt J, Boas U, Fenger M, Jakobsen MH.
Photochemical Immobilization of Anthraquinone Conjugated oligonucleoides and PCR Amplicons on solid Surfaces.
Bioconjugate Chem. 11 (2000), 474-483

2) Garcia L, Alonso-Sanz M, Rebollo MJ, Tercero JC, Chaves F.
Mutations in the rpoB gene of rifampin-resistant Mycobacterium tuberculosis isolates in Spain and the rapid detection by PCR-enzyme-linked immunosorbent assay.
J Clin Microbiol (2001), 39(5):1813-1818

3) Munch M, Nielsen LP, Handberg KJ, Jorgensen PH.
Detection and subtyping (H5 and H7) of avian type A influenza virus by reverse transcription-PCR and PCR ELISA.
Arch Virol (2001), 146 (1):87-97

4) Shamloul AM, Abdallah NA, Madkour MA, Hadidi A.
Sensitive detection of the Egyptian species of sugarcane streak virus by PCR-probe capture hybridization (PCR ELISA) and its complete nucleotide sequence.
(2001)

5) pUC 19 accession no: VB0026

6) Yanisch-Perron C, Vieira J, Messing J,
Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp 18 and pUC 19 vectors.
Gene, (1985) 33:103-119

Credits

Assay and protocol for Streptavidin plate preparation was designed by Kirsten Gerner-Smidt.

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