Application Note

Custom-made Thermo Scientific Nunc Immobilizer for DNA Binding

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Thermo Scientific Nunc Immobilizer DNA MicroWell plates and strips for covalent immobilization of aminated DNA can be custom-made upon request.

The production of the Nunc™ Immobilizer™ DNA surface introduces an ethylene glycol spacer and a stable electrophilic group that reacts with nucleophiles such as free amines. The spacer design and the density of electrophilic groups on this surface are optimized for detection of various types of molecules including aminated nucleic acids.

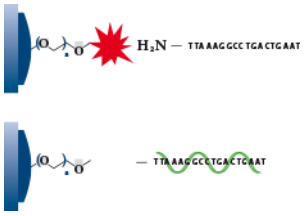

Fig. 1.

Binding mechanism for aminated nucleic acids

Coupling materials

- mmobilizer DNA MicroWell™ plate
- 100 mm carbonate buffer, pH 9.6
- Aminated PCR amplicon
- SSC (1 x SSC is: 150 mm NaCl, 15 mm sodium citrate, pH 7.0)
- PBST is Phosphate Buffered Saline 1 (PBS) with 0.05% (v/v) Tween 20

Coupling protocol

1. Prepare a solution of aminated DNA in 100 mm carbonate buffer, pH 9.6. It is recommended that the amount of aminated DNA is optimized, however for initial experiments we suggest: 1 nm aminated DNA PCR fragments (single stranded).
2. Add the aminated DNA solution to the wells of the Immobilizer DNA plate (100 µL/well) (50 µL for 384).
3. Incubate the plate with gentle agitation at room temperature for two hours or overnight at +4°C.
4. Aspirate the wells and wash with 3 x 300 µL 2 x SSC, 0.1% (v/v) Tween 20 (3 x 100 µL for 384).
5. The DNA surface is ready for use.

Detergents like Tween 20 effectively suppress covalent coupling of DNA and should consequently not be present in the coupling buffer. The use of competing nucleophiles like ethanolamine, lysine or tris (hydroxymethyl) amino methane (TRIS) should also be avoided in the coupling buffer.

The inclusion of small amounts of detergents like Tween 20 (0.05- 1% (v/v)) in subsequent wash and assay buffers, generally improves the signal to noise ratio of the assay. Other DNA concentrations, incubation times, temperatures, buffers or pH values than those recommended here can successfully be used.

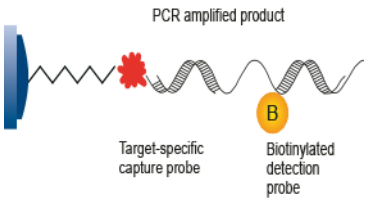

Fig. 1.

Detection of a PCR fragment by a target specific capture probe covalently linked to the DNA on an Immobilizer DNA plate using a biotinylated detection probe

Application examples

1. Detection of a PCR amplicon Using Immobilizer DNA for the detection of a PCR amplicon.

The DNA fragment to be detected was a 98 bp fragment from pUC19[2,3]. This 98 bp fragment was amplified using 5'- AAC AGC TAT GAC CAT

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G-3' and 5'-GTA AAA CGA CGG CCA GT-3' as primers, pUC19 as template and a standard PCR kit. The fragment was amplified following the manufacturer's recommendations and incubating: 2 min. at 94°C; 30 cycles (94°C 1 min., 45°C 1 min., 72°C 2 min.); and 72°C 3 min. The yield was estimated by agarose gel electrophoresis.

Protocol

1. Using the recommended coupling protocol, the capture sequence 5'-amine-AAC AGC TAT GAC CAT G-3' was covalently attached to the transparent Immobilizer DNA plate surface.
2. Per well: 10 µL of the PCR reaction (approximately 40 ng) was dissolved in 2 x SSC, 0.1% (v/v) Tween 20. Boiled 5 min. and then placed on ice.
3. The specific detection probe (5'-biotin-ATG CCT GCA GGT CGA C-3') was added to the PCR/SSC mix. 0.5 pmol detection probe per µL, final vol. 100 µL. The mix was then added to the wells of the Immobilizer DNA plate and the PCR fragment was allowed to hybridise to the covalently attached capture sequence for 3 hours at 37°C (Fig. 2).
4. The wells were aspirated and washed with 3 x 300 µL 2 x SSC, 0.1% (v/v) Tween 20.
5. A 1 µg/mL solution of streptavidin/HRP in PBST was dispensed into the wells (100 µL/ well), and the plate incubated for one hour.
6. The wells were aspirated and washed with 3 x 300 µL PBST.
7. A solution of 6 mm orthophenylene- diamine (OPD), 4 mm H2O2 in 100 mm citric acid buffer, pH 5.0 was added to the wells (100 µL/well) and left for color development.
8. After approximately 15 minutes, the enzyme reaction was stopped with H2SO4, 0.5 M (100 µL/ well) and the absorbance in this colorimetric assay was measured at 492 nm with an ELISA reader. The result are shown in Fig. 3. All incubations were carried out with gentle agitation at either room temperature or at 37°C when indicated.

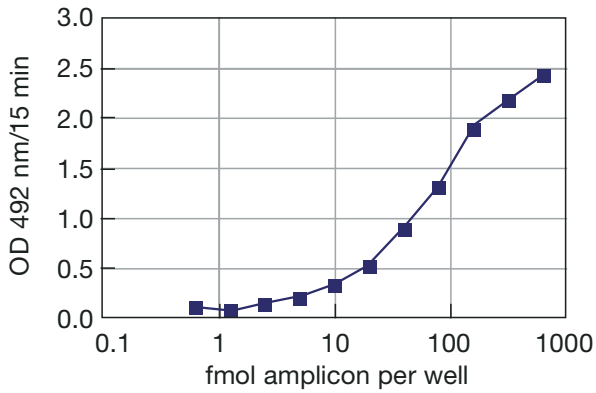

Fig. 3. Detection of various amounts of the 98 bp PCR fragment from pUC19

Special application using PCRproducts

With the advent of polymerase chain reaction (PCR), ligase chain reaction (LCR 4), and similar techniques, double-stranded (ds) DNA fragments with a well defined DNA sequence can be prepared. In particular the efficient generations of dsDNA fragments by PCR have found numerous applications in diverse fields of biomedicine and molecular biology.

Oligos with an amino group attached to its 5'-end can be purchased from most commercial oligo suppliers. Including one such primer in a PCR (or LCR) reaction result in the synthesis of aminolabeled dsPCR fragment. Such fragments can easily be covalently linked to the surface of the Immobilizer DNA plates and strips and used for various applications.

Enzymatic activity

A number of important enzymes, for instance restriction enzymes, kinases, phosphatases, polymerases, methylases etc. act on DNA. The possible relation between enzymatic activity and specific DNA sequences can conveniently be tested on DNA's covalently linked to the Immobilizer DNA surface. Analysis of DNA binding proteins We suggest that dsDNA's with various recognition sequences are generated and covalently attached to the Immobilizer DNA surface.

Gene discovery

A number of gene discovery methodologies (e.g. differential display 5) result in a large number of PCR fragments that have to be screened for the presence of a given consensus sequence. We suggest to attach such PCR fragments generated with one aminolabeled oligo to the surface of the Immobilizer DNA and screen for the presence of a particular DNA sequence as described below.

Immobilization and detection of an amino-labeled PCR amplicon on the surface of Immobilizer DNA plate.

To illustrate various aspects of the performance of the Immobilizer DNA plates for detection of an amino PCR amplicon. The DNA fragment to be detected was a 630 bp fragment from human Nras 6,7,8. This 630 bp fragment was amplified using 5'-NH2-C6- spacer-CCA GCT CTC AGT AGT TTA GTA CA-3' (position 1427-1449) and 5'-AAG TCA CAG ACG TAT CTC AGA C-3' (position 2035-2056) as primers, human Nras as template and a standard PCR kit. All oligos were purified by HPLC. The fragment was amplified following the manufacturer's recommendations and incubating 3 min. at 95°C; 30 cycles (55°C 2 min., 72°C 3 min., 95°C 1 min.); 55°C 2 min. and 72°C 3 min. The yield was estimated on a standard 1% agarose gel stained with ethidium bromide.

2. Detection of amino PCR amplicon

1. Using the recommended coupling protocol described above for a 96 well plate, the NH2- Nras amplicon was covalently attached to the Immobilizer DNA plate. 10 µL of the PCR reaction (approximately 120 ng) was diluted in 1:2 dilutions in 100 mm carbonate

buffer, pH 9.6, and 100 µL was dispensed per well. Incubation for two hours was allowed.

2. After coupling the amplicon was denatured by 200 µL 0.4 M NaOH 0.25% (v/v) Tween 20 for 5 minutes and washed with 3 x 300 µL 2 x SSC, 0.1% (v/v) Tween 20. The Nras DNA was detected by hybridization with the specific detection probe 5'-TGT GTT TGT GCT GTG GAA GAA CCCbiotin- 3' (position 1549-1572). The probe was diluted in 2 x SSC, 0.1% (v/v) Tween 20 final concentration. 100 µL 0.5 µm probe was added per well of the Immobilizer DNA plate. The detection probe was allowed to hybridise to the covalently attached sequence for two hours at 37°C.
3. The wells were aspirated and washed with 3 x 300 µL 2 x SSC, 0.1% (v/v) Tween 20.
4. A solution of streptavidin/ HRP in PBST (1 µg/mL) was dispensed into the wells (100 µL/ well), and the plate is incubated for one hour. The wells were aspirated and washed with 3 x 300 µL PBST.
5. A solution of ortho-phenylenediamine (OPD), 6 mm and H_2O_2, 4 mm in 100 mm citric acid buffer, pH 5.0 was added to the wells (100 µL/well) and left for color development.

After approximately five minutes, the enzyme reaction was stopped with H_2SO_4, 0.5 M (100 µL/well), and the absorbance was measured at 492 nm using an ELISA reader.

All incubations are carried out with gentle agitation at either room temperature or at 37°C when indicated.

Results

The result of a typical experiment is shown in Fig. 4. The experiment indicates that at high concentrations of the amino amplicon the hybridization signal decreases to the background level. This effect appears somewhat similar to the 'high-dose hook effect' described for various immuno-assays [9-11] and emphasizes that an optimization of amino amplicon concentrations is necessary.

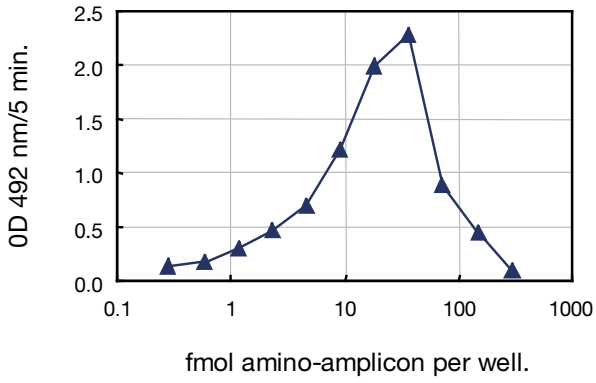

Fig. 4. Detection of various amounts of the 630 bp PCR fragments from Nras

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