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Covalent Binding of DNA to Thermo Scientific Nunc CovaLink NH Methods and Applications

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DNA can be bound covalently to Thermo Scientific Nunc CovaLink NH strips. The DNA molecules are bound exclusively at the 5'- end by a phospho-midate bond. Immobilization of more than 1 pmol is possible. When DNA coated Nunc™ CovaLink™ NH strips are used as the solid phase for hybridization assays, detection of less than $15 \cdot 10^{-18}$ moles is possible.

Introduction

Hybridization in Thermo Scientific Nunc MicroWell Plates is, due to their easy handling (multichannel pipettes, readers, and other instruments for ELISA techniques can be used), a very promising method for diagnostic purposes. Furthermore, MicroWell™ plates allow the use of non-radioactive probes.

So far, the limitations of hybridization in MicroWell plates have been the immobilization of DNA on the surface of MicroWells. Immobilization can be obtained by passive adsorption [1], by UV light [2-4], or by covalent binding of base modified DNA molecules [5-6]. These methods present the same disadvantages as for immobilization of DNA on nitrocellulose: i.e. the molecules are bound more or less efficiently to the solid phase at several sites.

The ideal immobilization of DNA on a solid phase is done by one single covalent bond, preferably at either the 3'- or 5'-end.

The use of CovaLink NH strips for covalent binding of DNA molecules at only the 5'-end has recently been described [7]. The DNA molecules were bound to CovaLink NH by a phosphoramidate bond [8].

CovaLink NH, a novel type of MicroWell surface, has secondary amino groups positioned at the end of spacer arms covalently grafted to the polystyrene surface through a »handle« (MW 200) (Fig. 1).

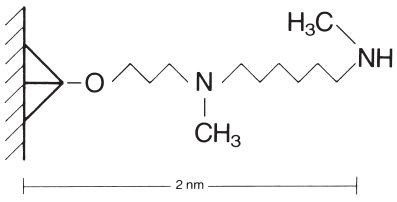

Fig. 1
The linker grafted onto CovaLink NH. The linker density is approximately 10^{14} / cm².

How DNA is bound to CovaLink NH, and the results obtained using CovaLink NH strips with covalently bound DNA molecules as solid phase for hybridization assays are described here.

Covalent binding of single stranded DNA (ssDNA) to CovaLink NH

Different types of DNA molecules have been used for binding (Fig. 2). All types were bound covalently to CovaLink Modules NH F8 (Cat. No. 478042).

It has been verified that the DNA molecules are bound exclusively at the 5'-end using non-phosphorylated ololigonucleotides for binding [7]. Non-phosphorylated oligonucleotides lack the 5'-end phosphate group, so they cannot be bound to CovaLink NH by the desired phosphoramidate bond. The use of non-phosphorylated oligonucleotides for binding reduced the amount of bound DNA molecules by 85%.

Method

DNA dissolved in water (7.5 ng/µL) was denatured for 10 min. at 95°C and cooled on ice (10 min.).

Ice-cold 0.1 M 1-methylimidazole pH 7.0 (1-MeIm$_7$) was added to a final concentration of 10 mm 1-MeIm$_7$. The ssDNA solution was dispensed into CovaLink NH strips (75 µL/well) standing on ice.

Carbodiimide (0.2 M) 1-ethyl-3-(3-dimethylaminopropyl)-

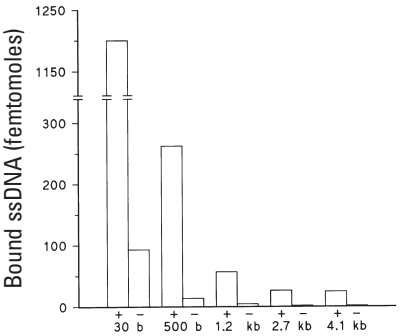

Fig. 2
Various types of DNA were used for binding. Oligonucleotide was phosphorylated with [γ-^{32}P] ATP, and 3 x molar excess ATP. λ DNA was digested with HinfI, resulting in fragments of different sizes. The majority of the fragments were 350-500 bp. Different plasmid DNA were linearized with HindIII or BgII + HindIII resulting in fragments of 1.2, 2.7, and 4.1 kbp. λ and plasmid DNA were 3' labeled with [α-^{32}P] dATP and Klenow enzyme. After coupling, the CovaLink NH strips were dissolved in toluene and the amount of bound DNA molecules was measured by liquid scintillation. Binding was made with (+) or without (–) carbodiimide.

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carbodiimide (EDC), dissolved in 10 mm 1-MeIm$_7$, was made fresh for each experiment, 25 µL was added per well. The strips were incubated for 5 hours at 50°C.

After incubation the strips were washed using Thermo Scientific Nunc Immuno Wash (e.g. Cat. No. 470174); first the wells were washed 3 times, then they were soaked with washing solution for 5 min., and finally they were washed 3 times. Washing solution: 0.4 N NaOH, 0.25% SDS heated to 50°C.

Hybridization of DNA immobilized onto CovaLink NH

The DNA molecules are immobilized on CovaLink NH at only the 5'-end. They are therefore ideal for hybridization. To test this a simple 2-layer technique was used.

Target DNA (plasmid DNA, 3.2 kbp) was linearized and coupled to CovaLink NH using the method described above. Target DNA was 1:2 diluted with non-target DNA of the same size. By using these dilutions for binding to CovaLink NH, the target concentration could be gradually reduced while the DNA concentration was kept constant.

Labeled probe (10^{16} cpm.^{32}P) was added to each well and hybridized overnight at 42°C.

Detection of $44 \cdot 10^{-18}$ moles target DNA bound per well was possible (Fig. 3).

Method

Prior to hybridization, the CovaLink NH strips were rinsed once with hybridization buffer (0.75 M NaCl, 5 mm sodium phosphate (pH 7.0), 5 mm EDTA, 0.1% Tween 20, 50% formamide, and 100 µg/mL sheared and denatured herring sperm DNA).

The strips were incubated overnight at 42°C in 100 µL hybridization buffer containing 10^6 cpm. probe per well. After incubation the strips were washed 3 times for 20 min. with 6 x SSC, 0.1% SDS at 60°C.

The wells were dissolved in toluene and the amount of hybridized probe was measured by liquid scintillation.

3-layer sandwich hybridization

CovaLink NH strips have been used for 3-layer sandwich hybridization. The strips were coated with capture probe (oligonucleotide, 30 b) and hybridized overnight with 1:2 dilutions of denatured target (plasmid DNA, 3.2 kb) and detection probe (biotinylated oligonucleotide, 30 b). Detection of the hybridization complex was done by addition of a fluorescent substrate.

With this method detection of less than $15 \cdot 10^{-18}$ moles of target DNA per well was possible (Fig. 4). Control hybridizations with $2 \cdot 10^{-15}$ moles of non-target DNA were performed (dotted line in Fig. 4).

Method

Approx. 10 ng capture probe was bound per well. After binding of capture probe, the strips were rinsed once with hybridization buffer (6 x SSC, 5 x Denhardt, 100µg/mL herring sperm DNA).

Hybridization was performed overnight at 45°C in 150 µL/well hybridization buffer with 1:2 dilutions of linearized target and $5 \cdot 10^{-15}$ moles/well detection probe.

After hybridization the plates were washed 3 x 20 minutes at 37°C, once with 2 x SSC, 0.1% Tween 20, and twice with 0.1 x SSC, 0.1% Tween 20.

After washing the strips were incubated with SA-AP conjugate, and 4-MUP was used as substrate.

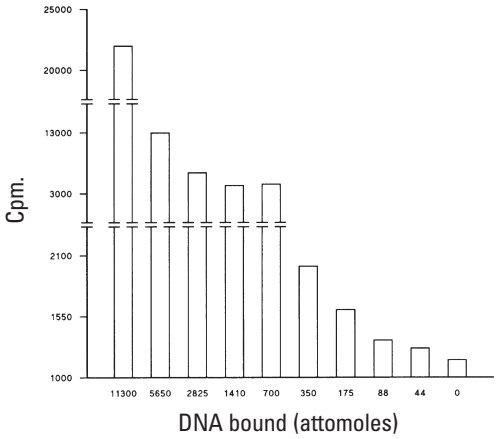

Fig. 3
Target DNA was 1:2 diluted with non-specific DNA of the same size. The DNA was bound to CovaLink NH as described. The modules were incubated overnight with hybridization buffer and 10^6 cpm. riboprobe per well. The wells were dissolved in toluene and the hybridized DNA was measured by liquid scintillation. The concentration of covalently bound DNA was approx. 11.3 fmol in all wells during hybridization, but the concentration of bound target DNA was 1:2 diluted from approx. 11.3 fmol to 44 amol.

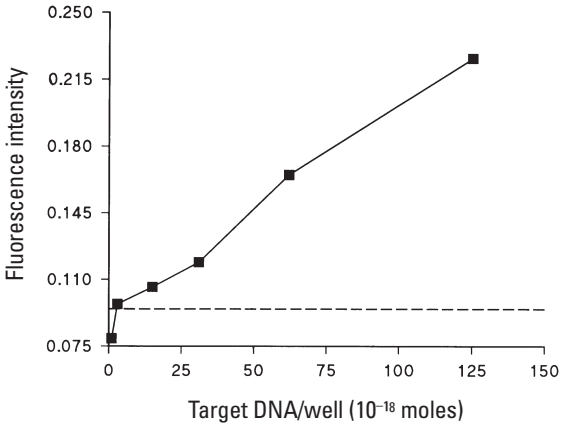

Fig. 4
Sensitivity obtained with 3-layer sandwich hybridization in CovaLink Modules. Approx. 10 ng capture probe was bound per well. Target DNA was added as 1:2 dilutions with biotinylated detection probe ($5 \cdot 10^{-15}$ moles/well).
After stringent washes, conjugate (streptavidin-alkaline phosphatase) was bound to hybridization complexes. Substrate was 4-MUP (4-methyl umbelliferylphosphate). The dotted line corresponds to the hybridization signals obtained when $2 \cdot 10^{-15}$ moles unspecific DNA was used as target.

Conclusion

DNA can be covalently bound to CovaLink NH strips almost exclusively by the 5'-end, which makes the use of CovaLink NH strips coated with DNA molecules very suitable for hybridization assays. The results with 3-layer sandwich hybridizations show detection of less than $15 \cdot 10^{-18}$ moles of DNA.

CovaLink NH strips have the format of MicroWell plates and can therefore be used in all known ELISA instruments, thus assuring easy handling and allowing the possibility of automation.

The combination of good hybridization results with the MicroWell plate format makes the CovaLink NH strip a very promising candidate for solid phase in DNA diagnostic assays.

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