Thermo Scientific Nunc NucleoLink Procedure for PCR ELISA

Key Words
Thermo Scientific™ Nunc™ NucleoLink™ surface, Thermo Scientific™ Nunc™ NucleoLink™ Strips, DIAPOPS, DNA binding, solid phase PCR, Elisa.

Goal

The goal of this application note is to give a detailed protocol for Solid Phase PCR ELISA using the NucleoLink surface.

The Nunc NucleoLink surface binds DNA covalently and heat-stably. The surface comes as our classical NucleoLink Strips for DNA hybridization applications as well as DIAPOPS.

For a protocol on solid phase PCR followed by detection by hybridization in the same well (DIAPOPS), please see Application Note 36, Nunc NucleoLink Procedure for Solid Phase PCR (DIAPOPS). This Tech Note gives a detailed protocol for PCR ELISA.

Preparation for and covalent binding of solid phase capture probe

1. The solid phase primer should be aminated or phosphorylated at the 5' end, and a linker of at least 10 T's (thymidine) should be added between the active primer sequence and the 5' end group.
2. Prepare a freshly made coating mix consisting of: 100 nm solid phase capture oligonucleotide and 10 mm EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) in 10 mm 1-methyl-imidazole (1-MeIm) (pH 7.0).
3. Add 100 µL of this coating mix to each Nunc NucleoLink well. This gives a total of 10 pmol of the 5´-phosphorylated or aminated solid phase oligonucleotide per well.
4. Seal the Nunc NucleoLink Strips (e.g. Nunc Sealing Tape).
5. Incubate the Nunc NucleoLink Strips at 50°C for 4-24 hours.
6. Wash the empty Nunc NucleoLink wells three times, soak for 5 minutes and wash three times, all with 100 mm Tris-HCl (pH 7.5), 150 mm NaCl and 0.1% Tween 20 at room temperature (RT). Empty the strips.
7. Wash the empty Nunc NucleoLink wells three times, soak for 5 minutes and wash three times, all with deionized sterile water.
8. The empty, coated Nunc NucleoLink Strips can be stored at 4°C or below in a polyethylene bag.

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Amplification

1. The amplification should be made as usual in traditional PCR tubes.

2. In order to label the PCR product, the amplification should be made using either:

a) Biotinylated primers (one or both PCR primers can be labeled), or

b) Addition of DIG-11-dUTP (a digoxigenine labeled oligonucleotide) at a concentration of 4 µm. The concentration of dTTP should be lowered to 0.125 mm. All other concentrations remain unchanged.
If non-labeled PCR products are detected, a labeled probe, complementary to the same strand as the solid phase capture probe, should be added during hybridization.

Detection

1. Add 10 µL of the PCR product to the Nunc NucleoLink wells in which the solid phase capture probe is covalently bound.

2. Add 10 µL of 1 M NaOH with 0.5 mg/mL thymol blue. This liquid is dark blue.

3. Incubate for 10 minutes at RT.

4. To each well, add 80 µL of 6.25 x SSC, 0.625% blocking reagent (BR), 0.125% Tween 20 and 0.5 M NaH_2PO_4 adjusted to pH 6.5 with NaOH. The pH of this mixture is 7.5, and the liquid should become yellow. If the color is red (acidic) or blue (alkaline), the hybridization will not be successful.

5. Incubate for 30 minutes-2 hours at 50°C (each system should be optimized individually).

6. Wash the NucleoLink wells three times at RT with 0.5 x SSC and 0.1% Tween 20.

7. Soak for 15 minutes at 50°C with 0.5 x SSC and 0.1% Tween 20.

8. Wash three times at RT with 0.5 x SSC and 0.1% Tween 20.

9. Detection of biotin-labeled PCR product

a) When using Alkaline phosphatase (AP): Add to each well 100 µL AP conjugated streptavidin diluted 1:3000 (or as the producer suggests) in 100 mm Tris-HCl (pH 7.5), 150 mm NaCl, 0.1% Tween 20 and 0.5% BR.

b) When using Horse Radish Peroxidase (HRP): Add to each well 100 mL HRP conjugated streptavidin diluted 1:5000 (or as the producer suggests) in 100 mm Tris-HCl (pH 7.5), 150 mm NaCl, 0.1% Tween 20 and 0.5% BR.

10. Detection of digoxigenin labeled PCR product
Add to each well 100 µL anti-DIG conjugated AP diluted 1:5000 in 100 mm Tris-HCl (pH 7.5), 150 mm NaCl, 0.1% Tween 20 and 0.5% BR.

11. Incubate for one hour at 37°-50°C sealed with Sealing Tape.

12. Wash the empty Nunc NucleoLink wells three times, soak for 5 minutes and wash three times with 100 mm Tris-HCl (pH 7.5), 150 mm NaCl and 0.1% Tween 20 at RT.

13. Two substrates have been tested with AP; 4-MUP (4-methylumbelliferyl phosphate) and pNPP (para nitrophenyl phosphate). One color forming reagent has been tested with HRP; TMB (3,3',5,5'-tetramethylbenzidine) in a ready-to-use solution.

a) When using 4-MUP: Add 100 µL of 1 mm 4-MUP dissolved in 1 M diethanolamine (pH 9.8) and 1 mm $MgCl_2$ to each well.

b) When using pNPP: Add 100 µL of 1 or 10 mg/mL pNPP in 1 M diethanolamine (pH 9.8) and 1 mm $MgCl_2$ to each well.

c) When using TMB: Add 100 µL of the ready-to-use solution to each well.

14. Substrate incubation The Nunc NucleoLink Strips should be sealed with Sealing Tape when incubating for longer than 30 minutes.

a) When using 4-MUP, incubate at 37°-50°C in the dark for 30-60 minutes. To stop the hydrolyzation of 4-MUP, add 50 µL of 3 M K_2HPO_4.

b) When using pNPP, incubate at RT for 30 minutes (10 mg/mL) to 24 hours (1 mg/mL). Add 100 µL of 1 M NaOH to stop hydrolyzation of pNPP.

c) When using TMB, incubate for 30 minutes at RT. Add 100 µL 0.1 M H_2SO_4 to stop the reaction. Note: Re-hybridization is not possible after addition of acid.

15. To measure the signal

a) To detect of hydrolyzed 4-MUP, determine the signal in fluorescence plate reader: Excitation 360 nm, emission 450 nm (also if the reaction has been stopped with K_2HPO_4).

b) For detection of hydrolyzed pNPP, measure OD in a normal ELISA plate reader at 405 nm (also if the reaction has been stopped with NaOH).
For detection of TMB, measure OD in a normal ELISA plate reader at 450 nm after the reaction has been stopped with acid (if the reaction is not stopped with acid, the color is blue and can be measured at 655 nm).

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