Application Note

Thermo Scientific Nunc NucleoLink Procedure for Solid Phase PCR (DIAPOPS)

Key Words

Thermo Scientific™ Nunc™ NucleoLink™ strips, covalent attachment, heat stable surface, binding of oligonucleotides, solid phase PCR, detection, hybridization.

Goal

The goal of this application note is to give a detailed protocol for solid phase PCR followed by detection and hybridization in the same well (DIAPOPS).

The Nunc NucleoLink surface binds DNA covalently and heat stably.

The Nunc NucleoLink strips are V-shaped with flat bottoms and are compatible with thermal cyclers and plate readers. Nunc NucleoLink can therefore be used for solid phase PCR followed by detection by hybridization in the same well (DIAPOPS).

The strips are available in transparent colorimetric detection of DNA. This application note gives a detailed protocol for DIAPOPS.

Preparation for and covalent immobilization of solid phase primer

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 primer 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´-modified solid phase primer per well.
4. Seal the Nunc NucleoLink Strips (e.g. with 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 once with deionized sterile water.
8. The empty, unsealed coated Nunc NucleoLink Strips can be stored at 4°C or below in an polyethylene bag.

Amplification

1. BSA must be added to the PCR mix in a final concentration of 1 mg/mL which should not be exceeded as this may block the solid phase amplification. 0.1%-0.25% Tween 20 is also recommended.
2. The primers must be added to the PCR mix in a ratio of 1:8. We use 25 pmol per reaction of the liquid phase primer and 25/8 pmol per reaction of the primer used as the solid phase primer.
3. Add PCR mix to the wells (normally 20 µL or 45 µL).

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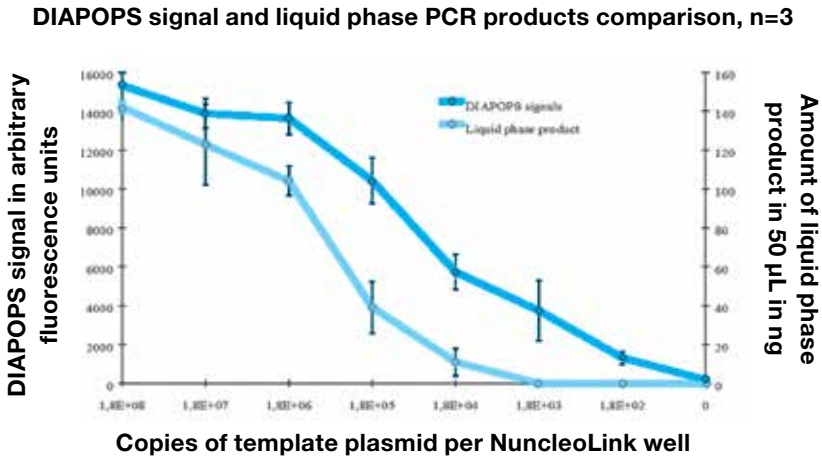
4. Add DNA template to each well (the total reaction volume has been tested with both 25 µL and 50 µL).
5. Seal the Nunc NucleoLink Strips with Sealing Tape 8.
6. Place the strips in a thermal cycler block.
7. Place the silicone spacer plate on the tape sealed Nunc NucleoLink Strips.
8. Temperature cycle the strips with the temperatures and cycling parameters specific for the system.
9. Remove the Nunc NucleoLink Strips and empty them.
10. Wash the empty Nunc NucleoLink wells three times, soak for 5 minutes and wash three times, all with freshly made 0.2 M NaOH and 0.1% Tween 20 at RT.
11. 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 RT.

Detection

1. Add 100 µL of 50-100 nm biotinylated hybridization probe diluted in 5 x SSC, 0.1% Tween 20 and 0.5% blocking reagent (BR) to each well.
2. Seal the Nunc NucleoLink Strips with sealing tape and incubate at 45-50°C for one to 20 hours.
3. Wash the empty Nunc NucleoLink wells three times at RT with 0.5 x SSC and 0.1% Tween 20.
4. Soak for 15 minutes at 50°C with 0.5 x SSC and 0.1% Tween 20.
5. Wash three times at RT with 0.5 x SSC and 0.1% Tween 20.
6. Detection of biotin label on the hybridized probe:
   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.
   When using Horse Radish Peroxidase (HRP): Add to each well 100 µL 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.
7. Seal the wells with sealing tape and incubate for one hour at 37°-50°C.
8. 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.
9. Substrate incubation:
   When using 4-methylumbelliferyl phosphate: Add 100 µL of 1 mm 4-MUP dissolved in 1 M diethanolamine (pH 9.8) and 1 mm $MgCl_2$ to each well. Incubate at 37°-50°C in the dark for 30-60 minutes. Add 50 µL of 3 M K_2HPO_4 to stop the hydrolyzation of 4-MUP.
   When using p-nitropheryl phosphate: Add 100 mL of 1 or 10 mg/mL pNPP in 1 M diethanolamine (pH 9.8) and 1 mm $MgCl_2$ to each well. 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.
   When using TMB: Add 100 µL of the ready-to-use solution to each well. Incubate for 30 minutes at RT. Add 100 µL 0.1 M H_2SO_4 to stop the reaction. (Re-hybridization is not possible after addition of acid).
10. To measure the signal:
    Detection of hydrolyzed 4-MUP: Determine the signal in a fluorescence plate reader: Excitation 360 nm, emission 450 nm.
    Detection of hydrolyzed pNPP: Measure OD in a standard plate reader at 405 nm.
    Detection of TMB: Measure OD in a standard 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).

Fig. 1

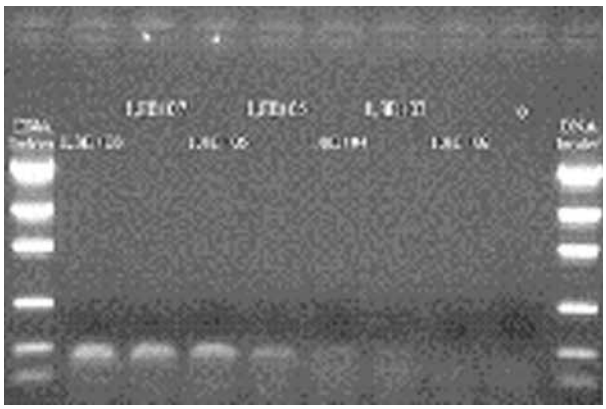


Preparation for re-hybridization or storage

1. Remove substrate from wells after measuring the catalyzed substrate.
2. Wash the empty Nunc NucleoLink wells three times, soak for 5 minutes and wash three times with freshly made 0.2 M NaOH and 0.1% Tween 20 at RT.
3. Wash the empty Nunc NucleoLink wells three times, soak for 5 minutes, and wash three times with distilled water or 100 mm Tris-HCl (pH 7.5), 150 mm NaCl and 0.1% Tween 20 at RT.
4. The washed empty Nunc NucleoLink Strips can be stored at 4°C or below in an ethylene bag.

Re-hybridization

1. No re-hydration of the Nunc NucleoLink Strips is necessary after storage. Commence with step 1 in the detection section and add hybridization solution directly to the empty dry wells.



Comparison of DIAPOPS and detection by gel electrophoresis. Using DIAPOPS the detection limit (1800 copies) was at least 1:10 improved compared to the gel (18000 copies).

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