

DIAPOPS using Thermo Scientific Nunc CovaLink BreakApart Modules

Key Words

Thermo Scientific™ Nunc™ CovaLink™ BreakApart Modules, PCR, covalent binding of oligonucleotides, EDC activating, detection of amplified products on a solid phase.

Goal

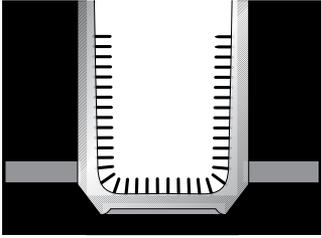
The goal of this application note is to describe the Detection of Immobilized Amplified Product in a One Phase System (DIAPOPS) method. This integrates amplification and subsequent characterization in the same well.

Polymerase Chain Reaction (PCR) has had a major influence on molecular biology and the development of molecular diagnostics. Despite the opportunities offered by PCR, the method has certain limitations. PCR can amplify DNA molecules a thousandfold, but the presence or absence of amplicons must be verified by other methods such as gel electrophoresis, Southern blotting, and various hybridization techniques.

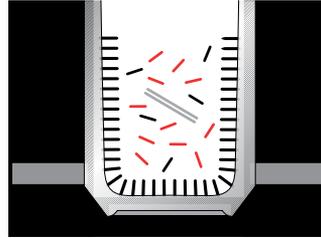
The necessity of characterization of the amplicons by other methods means time-consuming transfer. Also during transfer of the amplicons, laboratory contamination with trace amounts of amplicon might occur, wreaking havoc on subsequent amplifications. To reduce the possibility of contamination, techniques involving handling of DNA samples and amplicons in different rooms ¹, or using urasil DNA glycosidase to degrade contaminants ², have been used.



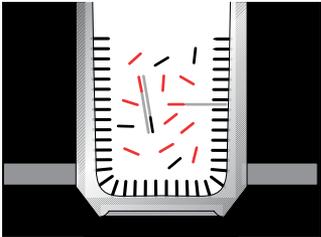
Principle of DIAPOPS



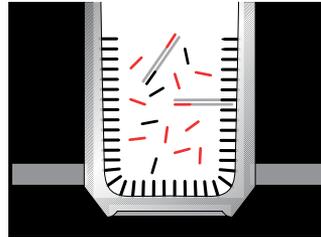
1. One primer is bound covalently at only the 5' end to the surface of a well. Either of the two primers can be used for binding. The choice between the upstream and the downstream primer depends on the oligonucleotide available as detection probe; the primer used for binding and the detection probe must be from opposite strands.



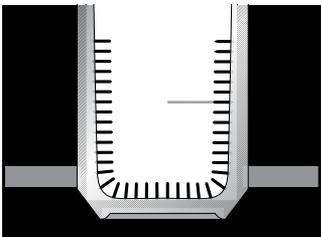
2. Buffer, dNTP's, Taq DNA polymerase, template and primers are added to a well containing the covalently bound primer. The amplification is semi-asymmetric since the relation between the bound and the unbound primer is 1:8.



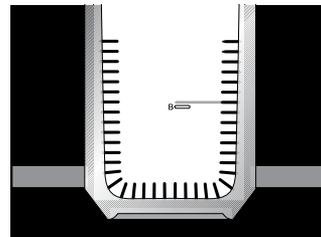
3. Amplification is initiated in the liquid phase. After approximately cycle 20, more and more amplicon will hybridize with the bound primer. These primers will be extended by the Taq polymerase.



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5. Amplicon in the liquid phase is removed by washing. The bound amplicon is converted to single stranded molecules by treatment with 0.2 N NaOH.



6. The single stranded molecules are detected by hybridizing with a detection probe. We used a biotinylated detection probe, but any type of detection probe can be used.

We have developed a new technique utilizing Thermo Scientific Nunc CovaLink BreakApart Modules. This technique, named DIAPOPS (Detection of Immobilized, Amplified Product in a One Phase System) integrates amplification and subsequent characterization³. The complete assay takes place in the same well.

DIAPOPS simplifies manipulations and reduces contamination, since no transfer of amplicon is needed. Here we describe DIAPOPS for detection of bovine leukemia virus (BLV).

Covalent binding of primer at the 5' end to Nunc CovaLink BreakApart Modules

To each well in a CovaLink BreakApart Module, 100 ng primer dissolved in 75 μ L 13 mm 1-methyl-imidazole pH 7.0 is added. Then 25 μ L freshly made 40 mm 1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide (EDC) is added, and the strips are incubated at 50°C for 5 hours. The total volume is 100 μ L and the final concentrations per well are 100 ng primer, 10 mm 1-methyl-imidazole and 10 mm EDC.

The use of 50 mm EDC for covalent binding of oligonucleotides to CovaLink NH Modules is described in the literature⁴. However, the use of 10 mm EDC during coating gives a better sensitivity for the DIAPOPS procedure.

After incubation the strips are washed at 50°C with 0.4 N NaOH, 0.25% Tween 20 using the Thermo Scientific™ Nunc™ Immuno™ Wash. First the strips are washed three times, then soaked for 5 minutes, and finally washed three times. Remaining wash buffer is removed by rinsing the strips once with deionized water.

The coated strips contain approximately 1 pmol covalently bound primer per well. The coated strips can be stored dry in a refrigerator for at least one year.

DIAPOPS

Amplification

5 μ L template DNA (5×10^{-23} - 5×10^{-16} mol BLV) was mixed with 45 μ L PCR buffer (10 mm Tris-HCl (pH 8.3), 50 mm KCl, 1.8 mm MgCl₂, 0.015% gelatin, 0.1% Tween 20), 200 μ M of each dNTP, 1 U Taq DNA polymerase, 0.5 μ M upstream primer and 0.06 μ M downstream primer (the downstream primer is also used for binding to the strips) and applied to each well of a coated strip. Evaporation was prevented by mineral oil.

Thermocycling

Cycle 1: 92°C for 5 seconds, 58°C for 1 second. Cycle 2-35: 91°C for 5 seconds, 58°C for 1 second. The fastest ramp time was used.

Since CovaLink BreakApart Modules are manufactured of polystyrene, they are heat labile. It is therefore important, especially during denaturations, to keep the cycling time as short as possible.

We used the MicroWell PlateCycler from Coy (Grass Lake, MI, USA).

This thermal cycler has two temperature probes, one

monitors the temperature in the heating block, the other mimics the temperature in the samples (sample probe).

During heating and cooling, the temperature profiles in the sample probe and the samples are not absolutely identical. However, the sample probe allows fast and efficient optimization of the amplification conditions.

Any thermocycler accepting 0.5 mL amplification tubes can be used. Break the strips apart and incubate the individual wells in the heating block. Oil may be applied to the heating block for a better heat transmission. After amplification, the wells can be replaced in the carrier and manipulated as a strip. However, when thermal cyclers other than the MicroWell PlateCycler are used, a less perfect fit must be expected. Therefore, the amplification procedures must be optimized for the particular thermal cycler accomplished. The optimization is most easily done by performing normal PCR in uncoated CovaLink BreakApart Modules and evaluating the amplicons by gel electrophoresis.

After amplification the strips are washed with 0.2 N NaOH utilizing the Immuno Wash. The strips are washed 3 times, then soaked for 5 minutes, and finally washed three times. After washing, the strips are rinsed once with hybridization buffer.

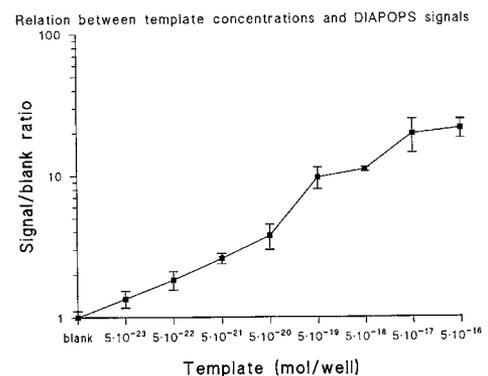
Hybridization and detection with fluorogenic substrate

Hybridization was performed overnight with 0.1 nm biotinylated detection probe at 45°C in 100 μ L hybridization buffer (2 X SSC, 5 X Denhardt's solution). After hybridization the strips are washed with 0.1 X SSC, 0.1% Tween 20.

The strips are washed three times, then soaked at 37°C for 10 minutes, and finally washed three times.

100 μ L diluted streptavidin alkaline phosphatase conjugate is added to each well, and the strips are incubated at 20°C for one hour. The streptavidin alkaline phosphatase conjugate is diluted 1:3000 with PBS, 0.05% Tween 20. After incubation, the strips are washed with PBS, 0.05% Tween 20 at 20°C as described above.

Finally, the strips are incubated at 37°C with 200 μ L 1 mm 4-methylumbelliferyl phosphate in 1 M diethanolamine (pH 9.8), 1 mm MgCl₂ for 30 minutes. The strips are read on a fluorescence plate reader: excitation wavelength 360 nm, emission wavelength 450 nm.



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ANZ: Australia: 1300 735 292, New Zealand: 0800 933 966; **Asia:** China Toll-free: 800-810-5118 or 400-650-5118;
India: +91 22 6716 2200, India Toll-free: 1 800 22 8374; Japan: +81-3-5826-1616; Other Asian countries: 65 68729717
Europe: Austria: +43 1 801 40 0; Belgium: +32 2 482 30 30; Denmark: +45 4631 2000; France: +33 2 2803 2180;
Germany: +49 6184 90 6000, Germany Toll-free: 0800 1-536 376; Italy: +39 02 95059 554;
Netherlands: +31 76 571 4440; Nordic/Baltic countries: +358 9 329 10200; Russia/CIS: +7 (812) 703 42 15;
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