Application Note

Versatile PCR Assays Based on Hybridization in Thermo Scientific Nunc MicroWell Plates

Lars P. Nielsen, Department of Clinical Microbiology, Aarhus University Hospital, 8000 Århus C, Denmark

Key Words
Thermo Scientific™ Nunc™ MicroWell plate, PCR, hybridization detection, Thermo Scientific™ Nunc™ NucleoLink™ strip

Goal

The goal of this application note is to highlight the advantages with detection of microbial pathogens using Nunc MicroWell plates and strips.

Assays based on the polymerase chain reaction (PCR) are used increasingly in clinical laboratories for the detection of microbial pathogens.

These tests are sensitive and able to detect agents that are difficult or impossible to culture. Only few commercial kits exist for the detection of microbial pathogens and often the laboratories have to develop PCR tests.

We have developed a versatile set of PCR assays with the following goals:

- Extraction procedure that can be used for most clinical samples
- One-step assays for both RNA and DNA targets
- Amplification controls that interfere minimally with target amplification
- Nunc MicroWell plate hybridization detection identical for all pathogen assays

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With this set of PCR assays we are able to detect infectious diseases.

In other circumstances the PCR assays are much more sensitive and rapid to perform than the classic clinical procedure. We are at present able to detect Legionella, Mycoplasma, Enterovirus, Herpes simplex virus, Varicella-Zoster virus, Adenovirus, Influenza A and B virus as well as respiratory syncytial virus in various clinical material.

The general assay is shown in Fig. 1, and an example of a visual reading is shown in Fig. 2.

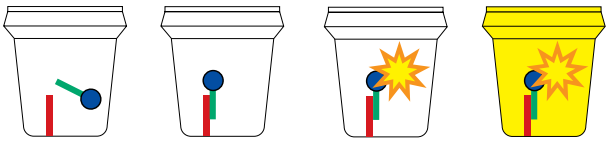

Fig 1. Assay development
A capture probe is covalently bound to the NucleoLink well. The biotinylated PCR product produced in a separate PCR vessel is denatured and captured onto the surface by the probe.

Added streptavidin conjugated to horseradish peroxidase binds to the biotin and is afterwards detected by the addition of a peroxidase specific colorimetric substrate.

Biotinylated PCR product

Capture probe

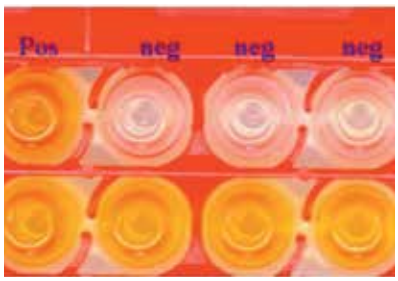

Fig 2. Solid phase hybridization in a NucleoLink Strip

The first row shows the hybridization result using a probe specific for the pathogen - in this case enterovirus. The second row shows the corresponding hybridization of the amplification of control DNA. Four samples were tested, and only the first sample is positive for the virus. All the hybridizations for the control DNA are positive.

Methods

Nucleic Acid extraction procedure

Prepare 400 µL of guanidinium thiocyanate (4 M) and dithiothethiol (1 mm) in TRIS buffer (10 mm, pH 5.6) per sample. The amplification control (RNA or DNA) is added to the guanidium buffer. Add 100 µL sample material.

Mix well. Add 500 µL isopropanol. Centrifuge at 10.000 g for 10 minutes at room temperature. Aspirate the supernatant. Add 750 µL 70% ethanol, mix and centrifuge as described above. Aspirate the supernatant. Air dry the sample. The amplification mix is added and the DNA is dissolved. Transfer to a PCR tube.

Amplification

DNA targets

Each pathogen undergoes 40 cycles with their specific parameters.

RNA targets

Incubation for 30 minutes at 42°C for the reverse transcriptase step. The RT enzyme is denatured and released from the nucleic acid by heating to 95°C for 60 seconds. Amplify as for DNA targets.

Detection of amplified material in Nunc NucleoLink Strips

1. Immobilization of capture probe

5´-phosphorylated probes are linked covalently to Nunc NucleoLink Strips. To each well, add 100 µL probe (100 nm) in freshly made 10 mm 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC) in 10 mm 1-methylimidazol (1-MeIm) (pH 7.0). Incubate for 5 hours at 50°C. Wash three times with 100 mm Tris-HCl (pH 7.5), 150 mm NaCl and 0,1% Tween 20. Wash with distilled water three times.

2. Capture of PCR products in NucleoLink Strips

Both primers used for the PCR assays are biotinylated at the 5' end.

The amplified material is denatured by adding an equal volume of 1.6% NaOH. 100 µL hybridization buffer (1 M phosphate buffer, pH 5.6) is added to each NucleoLink well. 25 µL of the denatured PCR product is added per well. Incubate at 37°C for 60 minutes. Wash three times with PBS containing 0.5% Tween 20 and add 100 µL streptavidin conjugated to horseradish peroxidase. Incubate for 15 minutes. Wash as described above and add TMB (3.3´, 5.5´ tetramethylbenzidine) substrate. After 10 minutes, 100 µL 0.1 M sulphuric acid is used to stop the color development. The result is read at 450 nm in an ELISA reader.

The construction of amplification controls

DNA controls

The primer sequence for the pathogen is added to the 5' end of a specific CMV (cytomegalovirus) sequence. After amplification the PCR product is used as an amplification control. To avoid competition between control DNA and target DNA from the pathogen, a mutation is introduced in the primers for the control. This leads to a higher affinity of the primers for the pathogen increasing the sensitivity of the assay as shown in Fig. 3.

RNA controls

As above except that a RNA polymerase specific control is produced by adding a RNA promoter sequence to one of the primer sequences. Transcription is done using T7 RNA polymerase.

Contaminating DNA is removed by RNase free DNase. Mutations are introduced as described.

For both the DNA and the RNA control a specific hybridization for CMV is performed in a well coated with a specific CMV probe.

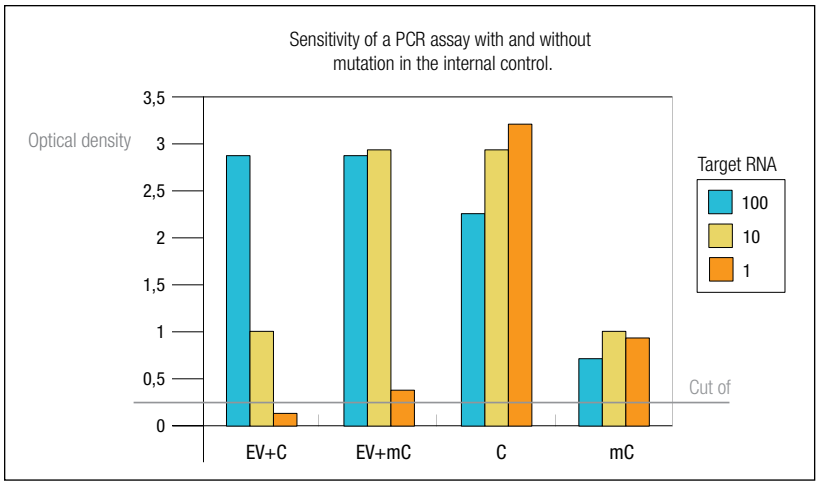

Fig 3. Comparison of the sensitivity of enterovirus (EV) PCR with a non-mutated (C) and a mutated control (mC)

Three samples of enterovirus were prepared by diluting the virus 1:10 in buffer. The first three bars show the sensitivity of the assay with a non-mutated control. It is clearly seen that the control competes with the control RNA (bar 7-9). When mutations are induced in the control RNA a higher sensitivity of the assay is observed (bar 4-6), and at the same time lower optical density in the control wells is seen. The inhibition of the assay is less pronounced using the mutated controls.

Discussion

The described PCR concept has, in our hands, proved to be working in a clinical microbiological laboratory, and the described goals have been accomplished. The reagents for the PCR/RT-PCR are mixed and divided in aliquots and stored in the freezer. The only differences between the assays lie in the control nucleic acid, the PCR or RT-PCR mix and the capture probe bound to the NucleoLink hybridization wells.

As can be seen in Fig. 3, the assay is very sensitive. With a mutation in the control it is possible to detect down to one copy of the target RNA, and the use of amplification controls secures that the reagents and extraction procedure are working properly.

ANZ: Australia: 1300 735 292, New Zealand: 0800 933 966; **Asia:** China Toll-free: 800-810-5118 or 400-650-5118;
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