Clean Pathogen DNA and RNA Kit: Pathogen detection in cow fecal samples

Abstract

Traditional Microbiology methods such as determination by pour plating and the morphological of physiological traits of micro-organisms have long been limited to the use of cultivation methods. However, Molecular Biology techniques eliminate the need for cultivation of potential pathogen samples, which is a good development especially since classical techniques are laborious and time consuming.

Molecular Biology techniques offer the convenience of principles such as Real Time PCR (RT-PCR) as well as Quantative PCR (qPCR). Due to the increased implementation of both RT-PCR and qPCR, there is an increased demand for Pathogen DNA isolation methods. To meet this demand, CleanNA[4] offers the Clean Pathogen DNA and RNA Kit[1,4], designed for high throughput and reliable isolation, high quality host genomic DNA, gram positive and negative bacterial DNA, fungal spore DNA, and viral DNA and RNA from tissue, urine, serum, and fecal samples. The Clean Pathogen DNA Kit allows for the processing of 1 single sample up to 96 samples, simultaneously providing high quality DNA and RNA with excellent purity.

The isolated DNA and RNA is directly suitable for downstream use for RT-PCR and/or qPCR assays enabling fast and reliable pathogen detection. With this application note we describe the isolation and detection of Pathogens in fecal samples from cows as performed at the Wageningen Bioveterinary Research[5] in Lelystad, The Netherlands. Pathogen DNA and RNA is first isolated and purified from the fecal samples using the Clean Pathogen DNA and RNA Kit, followed by a series of qPCR reactions to determine the pathogen species.

Introduction

With the rapid increase of adaption of molecular biology techniques in diagnostics in general and especially in microbiology as well as research, there is an increased demand for Pathogen DNA and RNA isolation methods. Disease-causing micro-organisms, such as bacteria, fungi and viruses can be found commonly in tissue, serum, fecal and urine samples. Most micro-organisms (pathogens) live off the host and can cause diseases as an indirect result of obtaining food from, shelter in, or spreading toxins in the host. Microbiology techniques are developed to detect pathogens in case of illness or preventatively to screen flocks of for example cattle and humans.

In DNA, the genetic information is stored for all cellular processes. The genetic code for these proteins is stored in conserved regions of the DNA (genes) code. Due to their essential role in cellular processes the structure of RNA coding genes is similar in all organisms. Most of the phylogenetic analysis performed nowadays makes use of the information stored in the 16S RNA molecule.

Conserved regions are found across different taxa or even across the eubacterial kingdom.

Wageningen Bioveterinary Research (BVR) operates in close collaboration with both government as well as companies. Their main goal is to protect the wellness and health of both humans and animals, by means of prevention, diagnostics and counseling.

Throughout the years, the BVR[5] has become the national reference institute for animal diseases. As a result of this extensive knowledge, it is the obligation of the BVR to determine the final diagnosis for animal diseases.

The BVR has a unique laboratory setup, including BSL3 facilities, with high security and fully isolated to provide a safe work environment for the work with these pathogens.

At the BVR one of the sample types used for detection of pathogens are cow fecal samples. Pathogens such as bacteria, viruses and parasites are responsible for specific diseases can be found within for example fecal samples.
To meet the current requirements of the BVR, we offer the Clean Pathogen DNA Kit, designed for high throughput and reliable isolation of DNA and RNA from bacteria, viruses and parasites from various sample sources. The Clean Pathogen DNA Kit combines the CleanNA technology with our specially formulated buffer system to eliminate the binding of PCR inhibiting compounds, present within the samples, onto our magnetic particles. Following grinding and lysis, the DNA and RNA is bound to our CleanNA Particles surfaces.
The CleanNA magnetic particles are separated from the lysates by using a magnetic separation device. Following a few rapid wash steps to remove trace contaminants, the purified DNA is eluted from the CleanNA particles for downstream applications using an elution buffer.

Materials & Methods

Equipment

- Clean Magnet Plate 96-Well RN50 (P/N CMAG-RN50) 4
- Permagen™ 24x1,5 mL Magnetic Separation Rack (Article No# MSR24)
- Biospec™ mini Bead Beater
- Centrifuge
- Omni Mix Homogenizer
- Heated shaker incubator (set to 70°C)
- Vortexer

Chemicals

- Clean Pathogen DNA Kit1,4, 96 preparations. Contains all necessary buffers, reagents, magnetic particles and Clean Disruptor plate. (Article No# CPT-D0096 or CPT-D0384)
- Nuclease free water
- 100% ethanol
- qPCR primer sets
- qPCR reaction mix Q
- qPCR reaction mix S

Labware

- ABgene AB0661 Deep Well Storage Plate
- 2 mL Screw cap Tubes
- 2 mL Eppendorf Tubes

Protocol Overview

Cow fecal samples are collected without the addition of any conservation buffers. At the start of the process the glass beads from the Clean Disruptor Plate individual tubes are transferred into individual 2 ml screw cap tubes. For the reliability of the test, the screw cap tubes guarantee that no cross contamination can occur during the homogenization process.
250 µL stool sample and 275 µL Lysis Buffer CPT are added to each individual tube. Once the screw cap of each tube is closed, the tubes are mixed using the Biospec™ Mini Bead Beater at full speed for 4 minutes. This homogenization step is crucial to break up the fecal sample and to crack the cell walls of Mycobacteria if present in the sample.
To prevent any cross contamination by aerosols, all sample tubes are being centrifuged for 1 minute at 2,000 x g at room temperature.
To finalize the lysis process, 50 µL PK Buffer CPT and 20 µL Proteinase K Solution is added to each sample. After a short mixing using a vortex, the tubes are incubated at 70°C under continuous shaking to allow digestion of the samples.
Once the lysis step is finished, all tubes are centrifuged at 20,000 x g for 5 minutes and 300 µL of the cleared supernatant is transferred into a clean isolation tube (2 ml Eppendorf tube).
Then 300 µL Binding Buffer CPT, 300 µL CPT Prep Buffer and 20 µL CleanNA Particles CPT are added to each sample and mixed thoroughly.
After 10 minute incubation time required to bind the pathogen DNA to the CleanNA Particles CPT, the tubes are placed in to the Permagen™ Magnetic Separation Rack. This allows for the separation of the CleanNA Particles CPT from the solution. Once the CleanNA particles CPT have cleared from solution, the supernatant is removed and discarded by pipetting. The Pathogen DNA is bound to the CleanNA Particles CPT and can then be washed in 3 steps using the provided wash buffers.
Instead of a lengthy drying step after the ethanol washes to evaporate traces of ethanol, which is common in most magnetic bead based methods, the

specially coated CleanNA Particles CPT allow for a rapid water wash step to facilitate this. By adding 500 µL water to each sample, with the tubes still in the magnet device, and removing the water within 30 seconds all traces of ethanol are being removed. After this final water wash, the samples are eluted by re-suspending the particles in 50-100 µL elution buffer provided with the kit. Lastly after a final separation of the particles using the Permagen™ Magnetic Rack, the eluates are transferred into clean storage tubes away from the CleanNA Particles CPT ready for use in downstream applications.

In comparison, Pathogen DNA was isolated from the same samples using competitor B. Competitor B's sample was prepared manually by homogenizing 3 gram of sample material in an Omnimix™ beaker with 12 mL PBS. After homonization, 200 µL of the suspension was processed using a Ribolyser™ (shaking for 1 minute at 6 m/s^2) after addition of manufacturers B's lysis buffer. After the final disruption process, the samples were incubated over night at 60°C in a shaking incubator. The cleared supernatant was processed in the competitor B's automated extracting device.
Final goal of the application described in this application note is to test cow fecal samples for the presence of mycobacterium *M. avium* ssp. *Paratuberculosis*.

To compare the performance of the Clean Pathogen DNA Kit versus competitor B, pathogen DNA was isolated from 5 samples. Prior to isolation, each sample was split into 2 equal fractions, providing 5 fractions in duplo. For each sample 1 fraction was isolated using the Clean Pathogen DNA Kit and 1 fraction using competitor B's protocol.
A qPCR reaction was performed for the detection of *M. avium* ssp. *Paratuberculosis*[2,3] on all isolated samples using specific primers, in the knowledge of the target DNA being 70% GC-rich.
The qPCR was performed in duplo, using two different qPCR mixes from different suppliers:
1. qPCR reaction mix Q, figure 1 and 2
2. qPCR reaction mix S, figure 3 and 4

After analysis the data of both samples isolated using the Clean Pathogen DNA Kit were compared to the samples isolated using competitor B.

Each qPCR is performed in a 20 µL reaction volume, consisting of:
2 µL DNA eluate
400 nM primer
200 nM probe (6FAM-BHQ1 labeled)

Reactions are run on an Applied Biosystems platform, the ABI9500, according the following cycle protocol:
2' 95°C
10" 94°C
30" 60°C
Repeat step 2 and 3, 50-60 times

Results

As shown in figure 1, competitor B scores negative for the detection of *M. avium* ssp. *paratuberculosis* in the qPCR test. Within the set of 5 samples, 3 samples show inhibition (standard curve data not shown), making it impossible to detect the *M. avium* ssp. *paratuberculosis*.
The samples isolated using the Clean Pathogen DNA Kit, shown in figure 2, score positive for the detection of *M. avium paratuberculosis* in all 5 samples.

As shown in figure 3, competitor B. scores positive on 1 sample and negative on 4 samples for the detection of *M. avium* ssp. *paratuberculosis* in the qPCR test. Within the set of 5 samples, 2 samples show inhibition (standard curve data not shown), making it impossible to detect the *M. avium* ssp. *paratuberculosis*.

The samples isolated using the Clean Pathogen DNA Kit, figure 2, score positive for the detection of *M. avium* ssp. *paratuberculosis* in all 5 samples. For an overview of the results, see table 1.

Table 1. qPCR detection results *M. avium* ssp. *paratuberculosis*

qPCR Mix	Competitor B	Clean Pathogen DNA Kit
qPCR Mix Q	5x undetected (3x inhibition)	5x detected
qPCR Mix S	1x detected 4x undetected (2x inhibition)	5x detected

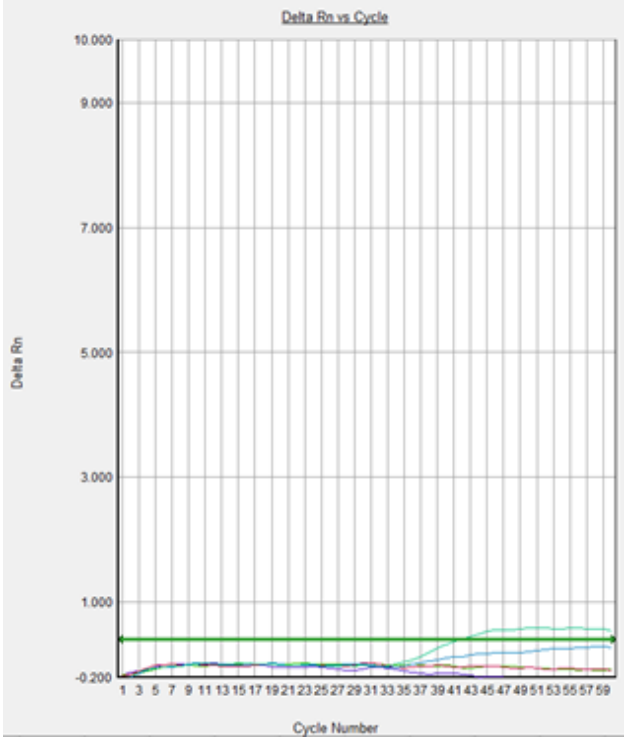

Figure 1. qPCR result on the 5 Competitor B isolated samples using the qPCR reaction mix Q.

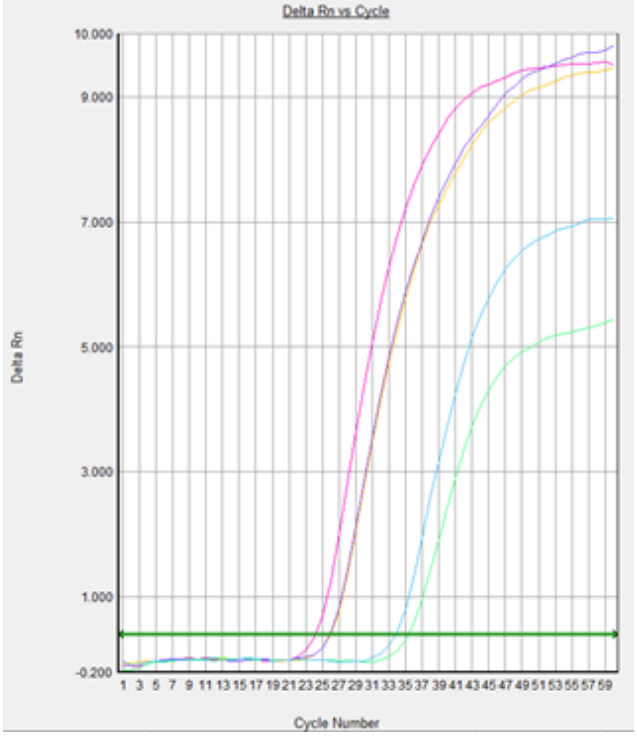

Figure 2. qPCR result on the 5 Clean Pathogen DNA Kit isolated samples using the qPCR reaction mix Q.

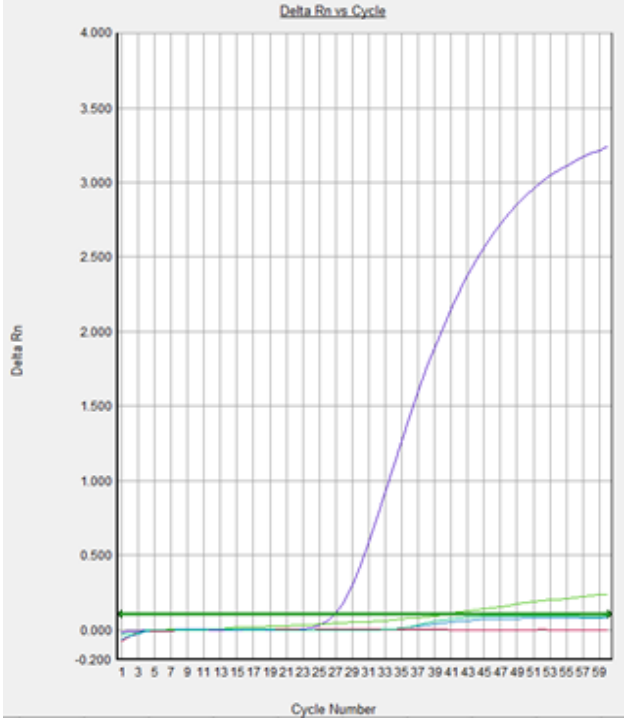

Figure 3. qPCR result on the 5 Competitor B isolated samples using qPCR reaction mix S.

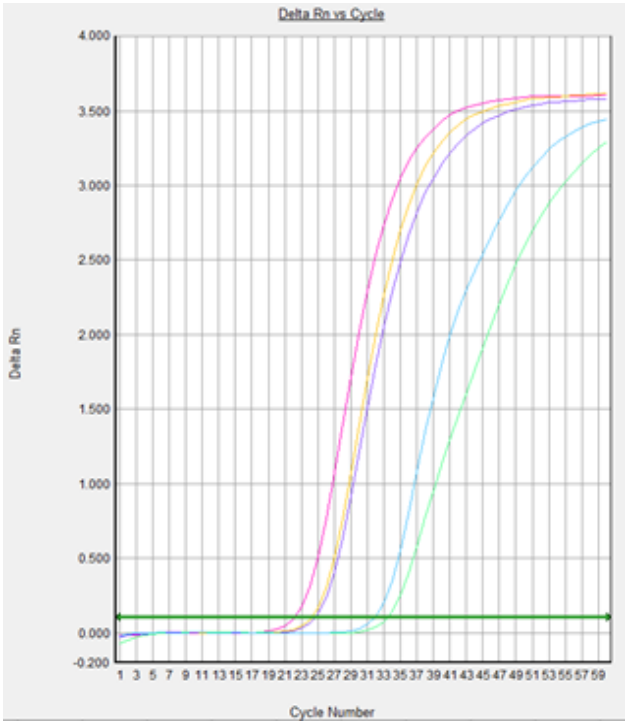

Figure 4. qPCR result on the 5 Clean Pathogen DNA Kit isolated samples using qPCR reaction mix S.

Conclusion & Discussion

The Clean Pathogen DNA Kit provides a lower Limit of Detection (LOD) as shown in the qPCR comparison with competitor B for the detection of *M. avium* ssp. *paratuberculosis*. This shows the DNA isolated using the Clean Pathogen DNA Kit is free of inhibitors, resulting in no false negative results.
The Clean Pathogen DNA and RNA Kit allows automation of the DNA and RNA isolation process from bacteria, viruses and parasites, significantly reducing the manual labor and minimizing the risk of cross-contamination and/or sample swabs.

This application note shows the performance of the Clean Pathogen DNA Kit used by the BVR in Lelystad. This has allowed the BVR to move away from current procedures and to massively increase the sensitivity of their qPCR tests resulting in a lower detection limit.

References

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