Using high volume *Superconvective* PCR to overcome PCR inhibition

Application Note #1

Large-volume Superconvective real-time PCR*, using the QuanTyper™-48 instrument, overcomes inhibitor induced amplification failure in bovine feces samples. This could have positive implications in situations where diagnosis of a disease is based on real-time PCR analysis of DNA originating from bovine feces, e.g. Johne's disease.

Introduction

Inhibitors present in clinical samples can reduce the sensitivity and specificity of diagnostic PCR assays and can give rise to false negative results. However, inhibitor concentration in a PCR reaction mixture can be reduced by dilution, either of the prepared DNA sample prior to amplification or by increasing the total reaction volume (1, 2), whilst keeping the sample volume constant. The former strategy reduces the number of target molecules available for amplification and is not recommended in cases where target copy numbers are initially low. The latter strategy, (which implies large reaction volumes) normally leads to slow thermal ramping, which may harm PCR efficacy. The upper reaction volume limit is therefore typically 100 µL in conventional instruments.

In this study we applied a novel strategy, *Superconvective PCR* (3, 4) that enables amplification in large sample volumes with fast temperature ramping. Using QuanTyper-48, a Superconvective real-time PCR instrument, we studied the amplification success of a low-copy number of a human gene sequence in the presence of a fixed amount of a bovine feces inhibitor background in volumes up to 200 µL.

Alpha Helix

Johne's Disease: A diagnostic challenge

Feces samples are difficult to analyze, partly because of the concentration of inhibitors such as bile salts and polysaccharides but also due to the presence of background DNA originating from host cells, organic matter in the food and from enteric bacteria. Additionally, high sample-to-sample variation in the composition and concentration of relevant compounds due to diet (2), physiological status of the animal, and sampling procedure, further complicates the analysis.

A typical example of a diagnostic problem is diagnosis of *Mycobacterium avium* subspecies *paratuberculosis*, the bacteria that causes Johne's disease in cattle (5). It is estimated that 20-30% of all herds in the USA are infected with this disease and the total economic loss, related to premature culling, decreased weight and value at slaughter and lower milk production, is estimated at 1.5 billion USD *per annum* in the USA alone (6).

The most commonly used method to diagnose Johne's disease is based on the culturing of fecal samples. This procedure can take up to 16 weeks to perform due to the extremely slow growth of the bacteria, particularly if only a few viable cells are present in the sample. During that period the infected animal continues to shed the bacteria, potentially infecting other animals in the herd. Thus, there is a great need for faster and more reliable diagnosis and numerous protocols based on the use of PCR have been developed. However, many of these protocols use dilution of the prepared DNA to overcome PCR inhibition. Such an approach may overcome inhibition but it is at the expense of sensitivity, thereby increasing the risk of false negative results. Here we show that by utilizing the high volume capability of the QuanTyper-48 instrument it is possible to overcome the negative effects of PCR-inhibition normally associated with DNA prepared from fecal samples.

Materials and Methods

Two DNA preparations were used for the experiments. One bovine feces DNA preparation was used as a background in qPCR reactions and one standard human DNA preparation was used as target for the amplification.

DNA was prepared from bovine feces using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. This kit includes a tablet for inhibitor removal. Initial amounts of feces were 100 mg for each preparation. The feces were collected from a single dropping from a healthy animal. Elution volumes used were 100 µL. DNA preparations used for comparative experiments were pooled before experimental setup to eliminate inter-preparation variation.

The pooled preparation is a non-specified mixture of DNA from bovine epithelial tissue, intestinal bacteria and herbal food content (1, 5, 6). It also included a host of inhibitors of different origins and chemistries (1) not subjected to further analysis. This preparation, having a DNA concentration of 15 ng/µL, was used as a standard DNA/inhibitor background in the experiment. The bovine DNA to human DNA ratios were 2750:1 for the 10% sample and 5500:1 for the 20% sample.

The PCR primers amplified a 115 bp fragment of the corticotropin releasing hormone receptor 2 gene in human genomic DNA and were used according to the reaction protocol (Table 1). In each PCR reaction, regardless of volume, 7.5 copies of human DNA were used as target template.

Table 1. Reaction protocol

	Final amount/concentration
Bovine fecal preparation (DNA/inhibitor background)	35/70 ng
Human DNA template	13 pg
Forward primer (5µM)	0.5µM
Reverse primer (5µM)	0.5µM
Platinum DNA polymerase mix[1]	1x
dH_2O	to final volume of 25-200µL

NB: The total amount of template DNA as well as competing/inhibiting bovine DNA preparation was kept constant.

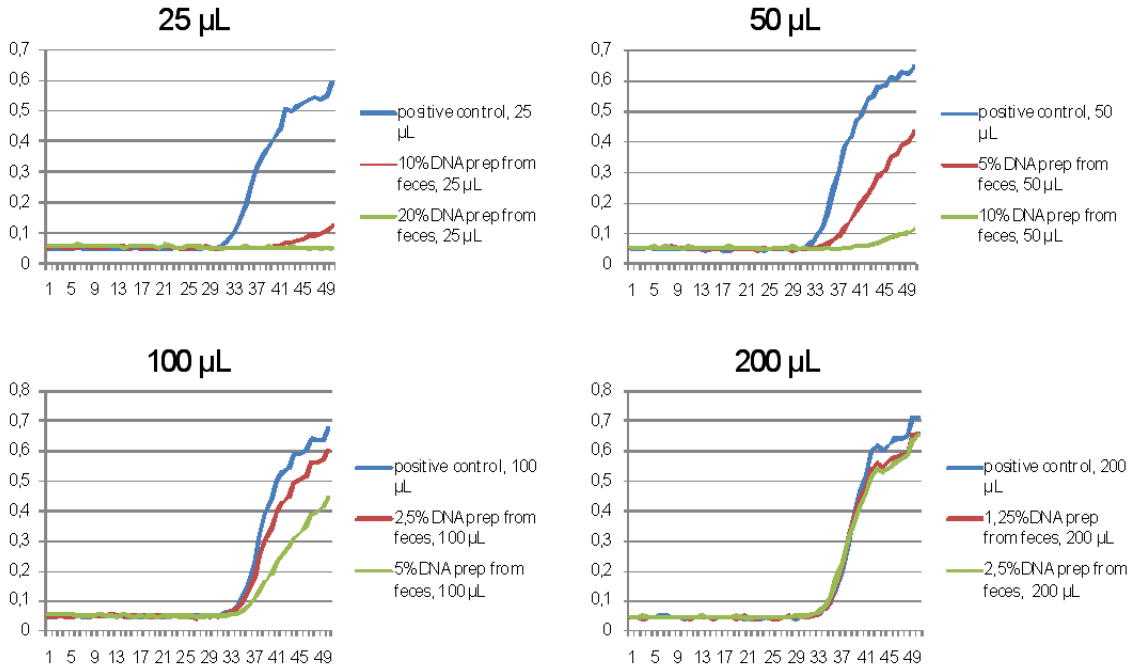
Cycling parameters
95°C for 90 s followed by 50 cycles of [95°C for 0 s; 58°C for 3 or 5[2] s; 72°C for 9 s].

[1]Platinum® SYBR® Green qPCR SuperMix-UDG with ROX is supplied at a 2x concentration and contains Platinum®*Taq* DNA polymerase, SYBR® Green I dye, Tris-HCl, KCl, 6 mM $MgCl_2$, 400 µM dGTP, 400 µM dATP, 400 µM dCTP, 800 µM dUTP, uracil DNA glycosylase (UDG), 1 µM ROX reference Dye and stabilizers. Invitrogen cat nr: 11744-100

[2]For 25 µL and 50 µL reactions an annealing time of 3 seconds was used, and for 100 µL and 200 µL reactions 5 seconds annealing time was used.

Results

As illustrated in the figure below, separate real-time PCRs were run in QuanTyper-48. The 25 µL PCR reaction was completely inhibited by the addition of 20% (v/v) of a DNA preparation from bovine feces (upper left graph). PCR amplification was rescued by gradually increasing the reaction volume, which dilutes the concentration of the target DNA template as well as the competing/inhibiting DNA. The 200 µL reactions (lower right graph) containing bovine DNA performed, essentially, as well as the positive control.



Conclusions

- *Superconvective PCR* technology enables DNA amplification in large sample volumes with fast temperature ramping.
- The QuanTyper-48 instrument employs superconvection to overcome the negative effects of PCR-inhibition and avoids the false negative results that are common with DNA prepared from fecal samples.
- The methodology is particularly interesting for situations where diagnosis of a disease is based on real-time PCR analysis of DNA originating from bovine feces, e.g. Johne's disease.

References

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*PCR is a patented technology owned by F. Hoffman-La Roche Ltd.