QuantiFast® Pathogen IC for evaluation of PCR inhibition

Lillian Krüger, Denis Flügge, Sandy Leifholz, and Holger Engel

The QuantiFast Pathogen PCR or RT-PCR +IC Kits together with the Rotor-Gene® Q cycler enable reliable identification of pathogen nucleic acids using real-time RT-PCR in the presence of an Internal Control (IC). There is no requirement for optimization. The Internal Control provided with these kits enables correct interpretation of results, considering inhibitory effects.

Introduction

Real-time PCR or one-step RT-PCR provides a reliable method for detecting viral RNA and DNA or bacterial DNA. The use of negative controls such as NTCs (no template controls) verifies positive results by ruling out contamination which causes false positive results. The verification of negative results requires controlling for any PCR malfunctions that may cause false negative results, such as incorrect PCR setup, thermocycler malfunction, or inhibition. Inhibition of reverse transcription and/or PCR is frequently observed when working with clinical samples. The most efficient way to monitor inhibition is the use of an IC, i.e., the simultaneous extraction and/or amplification of the pathogen target and an internal positive control in the same tube in a duplex reaction.

It is important that the IC is non-competitive with the pathogen target system with respect to the efficiency of target amplification. Another important design feature of an IC is the stability of the IC assay in comparison to the pathogen target assay. The IC must neither be too strong nor too weak to allow for correct interpretation of the inhibitory effects in a sample. If the IC assay runs more stably than the pathogen assay, this carries the risk of a dropout of the pathogen signal under inhibitory influence while the IC signal is still present, which would incorrectly indicate a negative result. Conversely, too weak an IC signal can lead to a high number of questionable results requiring re-testing. Therefore, only an ideal IC design and setup ensure the correct display of the degree of inhibition in a given sample relevant to the influence on the pathogen assay. Nonetheless, the IC assay should always be a little less stable than the pathogen assay to ensure that the IC assay will dropout before the pathogen assay in every case.

Here, we describe how the QuantiFast Pathogen IC can be applied for both control of nucleic acid amplification and for combined control of nucleic acid extraction and amplification. In addition, we demonstrate how the IC can be used to evaluate the inhibitory potential of PCR samples.

Materials and methods

Use of the QuantiFast Pathogen +IC Kits for control of nucleic acid isolation and PCR or RT-PCR amplification.

Six replicates of PBS and bovine whole blood were spiked with a fixed concentration of bovine herpes virus (BHV-1) and bovine viral diarrhea virus (BVDV). BHV-1 and BVDV virus particles were taken from cell culture supernatant. BVDV RNA and BHV-1 DNA were purified using the QIAamp® *cador*® Pathogen Mini Kit. For each pathogen, 3 of 6 purification replicates were processed after addition of IC DNA (High conc.) or IC RNA (High conc.) to the lysis buffer according to recommendations in the relevant QuantiFast Pathogen handbook (0.1 µl IC per 1 µl elution buffer). Duplex, real-time PCR or one-step RT-PCR was carried out with 10 µl of the eluates using the QuantiFast Pathogen PCR +IC Kit or RT-PCR +IC Kit without optimization according to kit handbooks. For each pathogen, IC DNA or IC RNA provided with the kit was added to the 3 of 6 replicates not containing IC DNA or IC RNA from the nucleic acid isolation procedure. Reactions were run on the Rotor-Gene Q real-time PCR cycler according to the protocol in the relevant handbook.

Indication of inhibition by the QuantiFast Pathogen IC

Viral RNA was isolated from BVDV cell culture supernatant using the QIAamp *cador* Pathogen Mini Kit and detected by real-time RT-PCR, using identical amounts of BVDV RNA in each reaction. Serial dilutions of solutions containing different types of inhibitory substance were either spiked into the reactions for simulation of increasing amount of inhibitors (fish sperm DNA, humic acid) or were processed using the QIAamp *cador* Pathogen Mini Kit before PCR (tissue homogenates, feces suspensions). In the latter case, selected tissue and feces loads were higher than recommended in the *QIAamp* cador *Pathogen Mini Handbook*.

For preparation of tissue-derived nucleic acid solutions, total nucleic acids were isolated from liver homogenates containing different starting amounts of bovine liver. For each dilution, 3 of 6 purification replicates underwent an on-column DNase digest. For preparation of fecal samples, a highly concentrated (20%) horse feces suspension was prepared and was then used for nucleic acid isolation with or without applying an appropriate pretreatment step (centrifugation at 14,000 x *g*).

Inhibitory solutions were spiked into reactions, and the IC RNA provided with the kit was added to the PCR reaction mix for control of reverse transcription and PCR amplification. Viral RNA was co-detected with IC RNA using the QuantiFast Pathogen RT-PCR +IC Kit according to kit handbook recommendations using a BVDV-specific primer-probe set without any optimization of the duplex setup. Reactions were run on the Rotor-Gene Q as described above.

Results

Use of the IC for control of nucleic acid purification and PCR amplification

The QuantiFast Pathogen PCR and RT-PCR +IC Kits enable the use of the IC for control of the PCR amplification step alone or for combined monitoring of the nucleic acid isolation procedure and PCR amplification. For RNA applications, the IC RNA also controls for successful reverse transcription, which permits subsequent cDNA amplification. The IC DNA or IC RNA solution provided with the kits is added to the PCR for control of PCR amplification. The IC DNA (High conc.) or IC RNA (High conc.) can be ordered separately for use in nucleic acid isolation procedures. These higher concentrated ICs allow the adaptation of the IC amount to varying elution volumes and PCR input amounts. Figures 1 and 2 show how the use of the IC DNA (High conc.) and IC RNA (High conc.) according to handbook recommendations results in similar C_T values in comparison to the IC DNA and RNA provided with the kits used for control of PCR amplification.

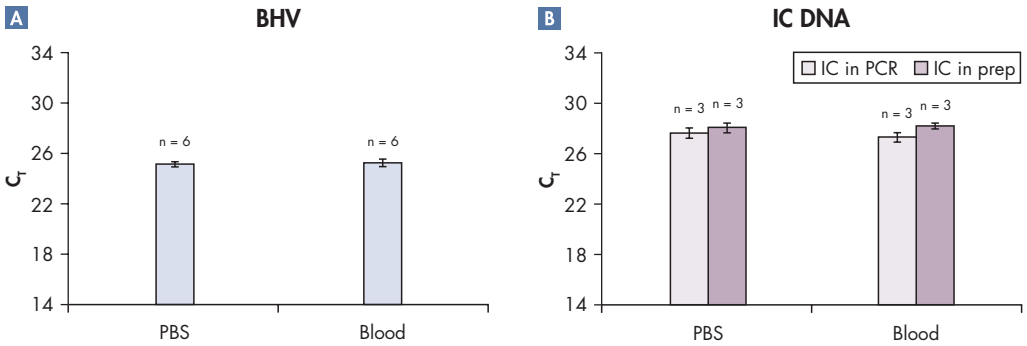

Figure 1. Flexible use of IC DNA. Comparison of addition of IC DNA for control of the PCR amplification ("IC in PCR") versus addition of IC DNA (High conc.) for combined monitoring of the nucleic acid isolation procedure and PCR amplification ("IC in prep"). IC DNA (400) provided with the QuantiFast Pathogen PCR +IC Kit was used. The IC DNA (High conc.) for the "IC in prep" (at the start of DNA isolation) experiment was added at a ratio of 0.1 µl per 1 µl of elution buffer. A C_T values obtained in PBS and whole blood samples for the BHV target in all 6 replicates. B C_T values for the IC DNA are shown for the amplification and extraction control. Error bars represent ±1 SD of the extraction replicates.

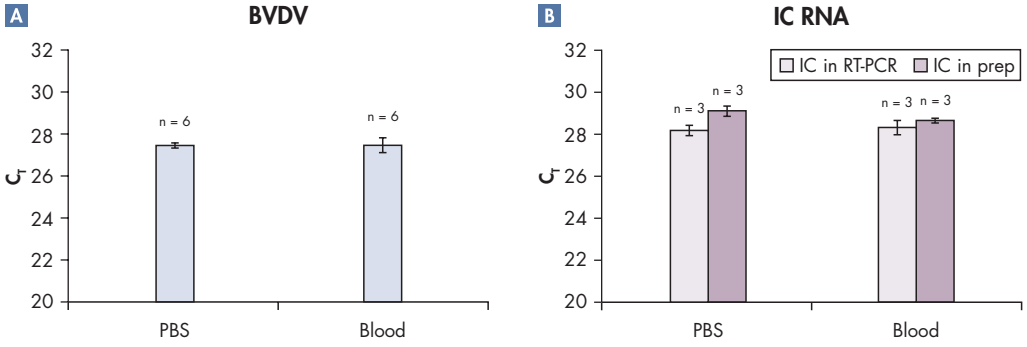

Figure 2. Flexible use of IC RNA. Comparison of addition of IC RNA for reverse transcription and PCR amplification ("IC in RT-PCR") versus addition of IC RNA (High conc.), at the start of the RNA isolation procedure, for control of RNA isolation, reverse transcription, and PCR amplification ("IC in Prep"). IC RNA (400) provided with the QuantiFast Pathogen PCR +IC Kit was used. The IC RNA (High conc.) for the "IC in prep" (at the start of RNA isolation) experiment was added at a ratio of 0.1 µl per 1 µl of elution buffer. A C_T values obtained in PBS and whole blood samples for the BVDV target in all 6 replicates. B C_T values for the IC RNA are shown for the amplification and extraction control. Error bars represent ±1 SD of the extraction replicates.

Tip: Use the same or a similar setup as described here for evaluation of the correct amount of IC (High conc.) tailored to your samples and extraction method. If the C_T value obtained for the IC (High conc.) after the nucleic acid isolation procedure differs more than ±2 C_Ts from the C_T value obtained after addition of the IC provided with the kit, adapt the amount of IC (High conc.) used for extraction. To protect the IC (High conc.) from degradation, do not add the IC directly to the samples but to the lysis buffer or lysate only.

Indication of inhibition

Due to its unique design and setup, the QuantiFast Pathogen IC accurately reflects the inhibition of the target system, as shown in the examples below.

Background DNA may negatively influence PCR. Principally, the reverse transcription step in RT-PCR applications may be impaired. Figure 3 illustrates the negative influence of high amounts of background DNA (fish sperm DNA) on RT-PCR performance. Both the viral RNA and IC are incrementally inhibited with increasing amounts of background DNA. The IC correctly indicates inhibition in relation to pathogen assay inhibition. With biological samples, inhibition of RT-PCR is often observed when samples contain a very high amount of host DNA, such as in tissue samples. The IC serves to detect the degree of inhibition in such samples (Figure 4). While the background DNA from the undiluted tissue homogenate strongly inhibits both the pathogen and the IC assay, the inhibitory effect decreases with increasing dilution of liver homogenates used for nucleic acid extraction. No inhibition is observed with the replicates of the same samples for which a DNAse digest was applied during the nucleic acid isolation procedure.

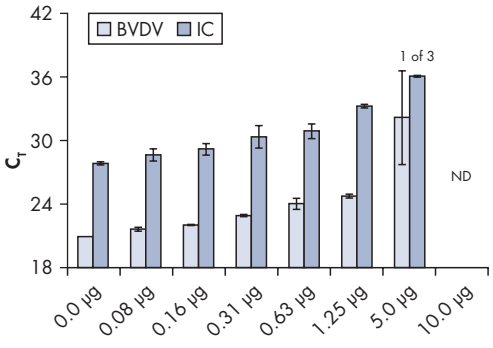

Figure 3. Increasing amounts of fish sperm DNA solution result in incremental inhibition of both the viral RNA target and the IC. With 5 µg fish sperm DNA in the reaction, the BVDV signal shows a shift of approximately 10 C_Ts and high variance of the 3 replicates tested. With the same concentration of fish sperm DNA, only 1 of 3 replicates of the IC is detected with a very high C_T while 2 replicates dropout completely. With 10 µg fish sperm DNA, both the BVDV and the IC assay are entirely inhibited with no signal being detected in real-time RT-PCR. Error bars represent ±1 SD of 3 PCR replicates. **ND**: Not detected.

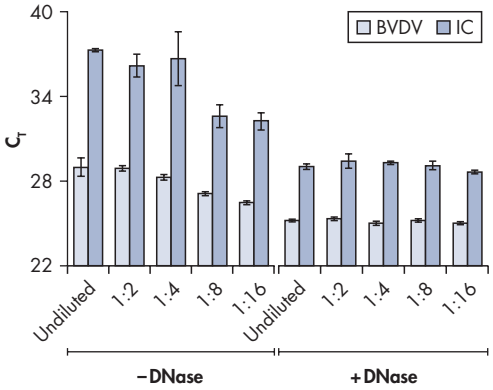

Figure 4. Very high amounts of total nucleic acids isolated from liver tissue result in inhibition of BVDV real-time RT-PCR detection. The inhibition is reflected by an increase of the C_T value for the IC. The inhibitory effect is attenuated by the use of lower amounts of liver homogenate in sample preparation. No inhibition is observed with the same samples when a DNAse digest was applied during the nucleic acid isolation procedure (+DNase). Error bars represent ±1 SD of 3 extraction replicates.

Some biological sample types, such as fecal samples can contain high amounts of inhibitory substances, e.g., humic acid, phenolic components, and other substances which may be carried over into nucleic acid eluates during sample preparation if no appropriate pretreatment is applied. These substances can inhibit PCR or reverse transcription. Figure 5 shows the C_T shifts of the pathogen and IC assay with increasing amounts of humic acid in the reaction with a dropout of the IC signal at a 500 pM concentration of humic acid. The IC dropout occurs just before pathogen assay failure at 600 pM concentration, again displaying the optimal function of the IC. Figure 6 shows the difference an appropriate pretreatment can make to results with inhibitory fecal samples. The C_T shifts of the pathogen target are correctly reflected by the IC.

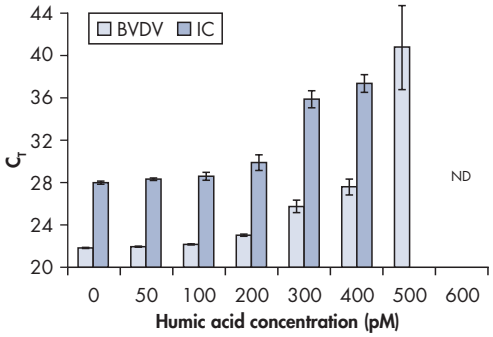

Figure 5. Increasing inhibition with increasing concentrations of humic acid. Following inhibition by humic acid, the IC assay drops out at a concentration of 500 pM humic acid, followed by the pathogen assay dropping out at 600 pM humic acid. Error bars represent ±1 SD of 3 PCR replicates.

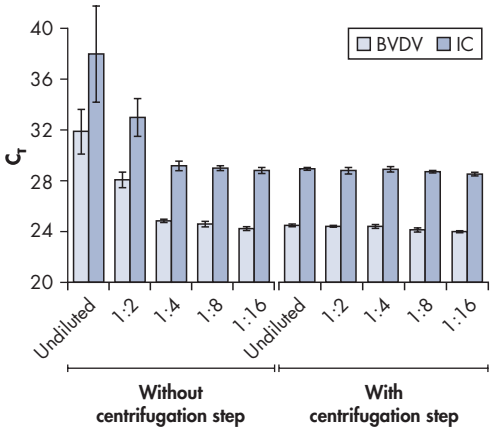

Figure 6. Better results for fecal samples using an appropriate pretreatment. A dilution series was prepared from a highly concentrated (20%) horse feces suspension and resulting suspensions were further processed with or without an appropriate pretreatment step (centrifugation at 14,000 x *g*). Undiluted and 1:2 diluted fecal suspension inhibit the BVDV RT-PCR where no pretreatment was applied (left panel). The IC correctly displays the degree of inhibition. Error bars represent ±1 SD of 3 extraction replicates.

Conclusions

- The QuantiFast Pathogen PCR and RT-PCR Kits provide reliable identification of viral RNA and DNA and bacterial DNA without the need for optimization of duplex set-up with the IC.
- Co-amplification of user defined pathogen target and IC ensures the correct interpretation of negative results. The IC can be flexibly used for control of extraction and/or PCR amplification.
- The IC design ensures an accurate reflection of the degree of inhibition of the target system which is displayed by shifts or drop outs of the IC signal.

Ordering Information

Product	Contents	Cat. no.
QuantiFast Pathogen PCR +IC Kit (100)	For 100 x 25 µl reactions: Master Mix, lyophilized Internal Control Assay, lyophilized Internal Control DNA, ROX Dye Solution, High-ROX Dye Solution, RNase-Free Water, Nucleic Acid Dilution Buffer, Buffer TE	211352
QuantiFast Pathogen RT-PCR +IC Kit (100)	For 100 x 25 µl reactions: Master Mix, RT Mix, lyophilized Internal Control Assay, lyophilized Internal Control RNA, ROX Dye Solution, High-ROX Dye Solution, RNase-Free Water, Nucleic Acid Dilution Buffer, Buffer TE	211452
QIAamp *cador* Pathogen Mini (50)	For 50 RNA/DNA preps: 50 QIAamp Mini Spin Columns, Carrier RNA, Proteinase K, Collection Tubes (2 ml), RNase-free Buffers	54104
QIAamp *cador* Pathogen Mini (250)	For 250 RNA/DNA preps: 250 QIAamp Mini Spin Columns, Carrier RNA, Proteinase K, Collection Tubes (2 ml), RNase-free Buffers	54106

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