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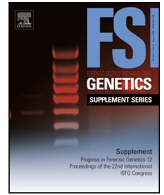

DTT quenches the passive reference signal in real-time PCR

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ABSTRACT

Forensic DNA analysis is partly limited by PCR-inhibitory compounds present in the DNA extracts. Generally, these inhibitors disturb amplification, i.e., the production of amplicons. We have found that dithiothreitol (DTT) from the DNA extraction process can cause another type of real-time PCR disturbance, i.e., inhibition of signal detection through fluorescence quenching. DNA extracts containing DTT substantially quenched the passive reference signal in the Quantifiler HP DNA Quantification kit. This quenching resulted in overestimation of DNA concentrations, as target DNA signals are normalized to the passive reference signal.

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1. Introduction

Forensic DNA analysis is partly limited by PCR-inhibitory compounds present in the DNA extracts [1]. Generally, these inhibitors disturb the production of amplicons by directly affecting the DNA polymerase, by changing the ion composition or by interfering with the target DNA [2]. Sidstedt et al. recently showed proof of principle of another type of inhibition, i.e., disturbance of the qPCR fluorescence signals [3]. There, humic acid quenched the fluorescence of DNA binding dyes such as EvaGreen and SYBR Green I, thereby interfering with amplicon detection.

During in-house validation of the Quantifiler HP DNA Quantification kit (Quantifiler HP) (Thermo Fisher Scientific), some DNA extracts received overestimated DNA concentrations. Looking into this more closely, quenching of the fluorescence signal of the passive reference dye was observed. The signal of the passive reference is used to normalize target signals in order to minimize well-to-well variation. Thus, lowered passive reference signals give higher normalized target signals. In this work we determine the cause and solution of this unexpected inhibition effect.

2. Materials and methods

During in-house validation of Quantifiler HP, several DNA extracts obtained from different extraction methods were quantified. Chelex based methods (Bio-Rad Laboratories) with the addition of 0.1 mg/mL Proteinase K (Sigma–Aldrich ref. no. P-2308) were mainly used. For semen and hair samples, 40 mM dithiothreitol (DTT) (Sigma–Aldrich ref. no. D-9779) was also applied. For these two sample types, both crude extracts and extracts purified with Amicon Ultra 2 mL filter devices (Merck Millipore) were quantified ($n = 6$). The extracts were quantified using Quantifiler HP (passive reference dye: Mustang Purple) and Quantifiler Human DNA Quantification kit (passive reference dye: ROX) on Applied Biosystems 7500 Real-Time PCR System with HID Real Time PCR Analysis Software v1.2 (Thermo Fisher Scientific). Extraction negative controls from each method were also analyzed. Apart from analyzing extracts, four different amounts of pure DTT were applied in Quantifiler HP reactions ($n = 9$): 0.033 mM; 0.33 mM; 3.3 mM and 33 mM.

3. Results and discussion

Generally, the DNA concentrations given by Quantifiler HP correlated well with the previous quantification system Quantifiler Human and the STR analysis kit PowerPlex ESX 16 Fast System (Promega Corporation). Quantifiler HP enabled precise quantification down to 0.003 ng/μL (data not shown). However, DNA concentrations were overestimated for crude semen and hair extracts. For example, one semen sample received a concentration of 0.4 ng/μL, compared with 0.1 ng/μL for Quantifiler Human, with STR analysis corroborating the latter result (data not shown). Some negative controls from these DNA extractions were falsely given high DNA concentrations (>50 ng/μL), although no amplification plots were visible (data not shown).

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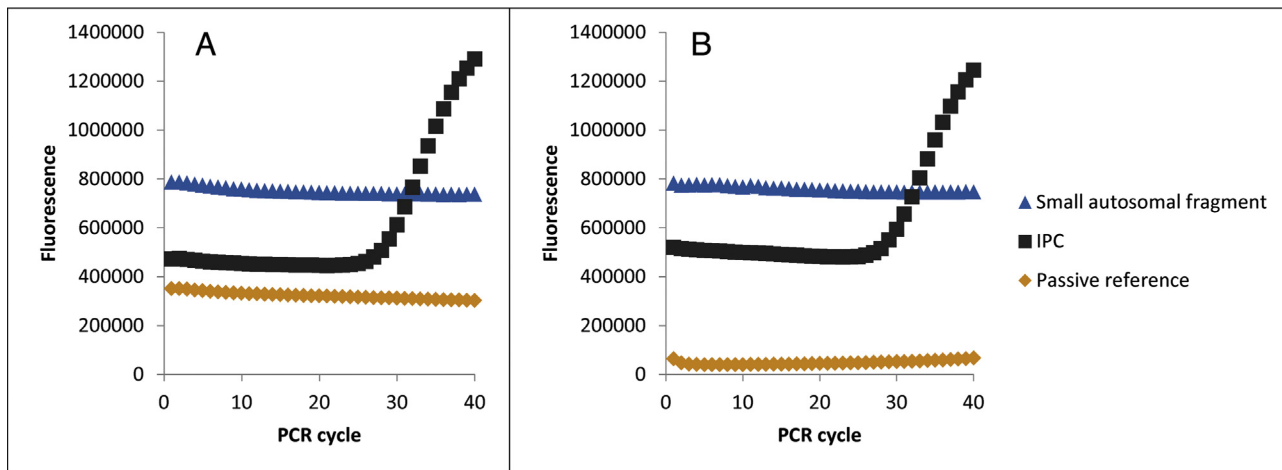

Fig. 1. Quenching of passive reference dye fluorescence by DTT. Fluorescence signals of the small autosomal fragment, IPC and passive reference are shown. (A) Extraction negative control without DTT, (B) Extraction negative control with DTT (~4 mM in the qPCR reaction).

Looking deeper into the data, the samples and controls showed clearly lowered fluorescence intensities for the passive reference dye (Fig. 1). The relative fluorescence intensity of the passive reference dye for negative controls from semen and hair extractions were about 10–20% compared with "normal" extracts (data not shown). No other fluorophores were affected. Thus, normalization against the lowered passive reference signal caused a false increase of reporter signals (ΔRn), making the amplification plots reach the threshold at earlier cycle numbers. The DNA extraction procedures for semen and hair have the addition of DTT in common, making this compound a plausible cause for the fluorescence quenching. When crude semen or hair extracts are analyzed the concentration of DTT in the quantification reaction is approximately 4 mM.

Pure DTT quenched passive reference dye fluorescence in a manner similar to crude semen and hair extracts, reinforcing that DTT causes the fluorescence inhibition. The relative intensity of the passive reference signal decreased with increasing amounts of DTT ($n = 9$): 0.033 mM (100% relative fluorescence intensity); 0.33 mM (70%); 3.3 mM (20%) and 33 mM (3%). The structure of the passive reference dye Mustang Purple is a trade secret, making it difficult to elucidate how DTT affects the dye. Being a strong reducing agent, DTT may change the structure of the dye and thereby disturb its fluorescence properties. The DTT effect is not general for passive reference dyes since the ROX dye used in Quantifiler Human is unaffected (data not shown).

The quenching effect caused by DTT disappeared when the semen and hair extracts were purified with Amicon Ultra filter devices prior to quantification (data not shown). A general issue is how to reveal passive reference dye quenching in casework. Amplification inhibition gives rise to elevated quantification cycle (Cq) values for the internal positive control (IPC) and can be monitored by applying an upper limit of acceptance for IPC Cq. In this study, about 80% of the reactions with pure DTT instead showed lowered IPC Cq values, due to the quenching of the passive reference signals (data not shown). Thus, applying a lower limit of acceptance for the IPC Cq could be a way of monitoring passive reference dye fluorescence inhibition.

Conclusions

DTT quenches the fluorescence of the passive reference dye Mustang Purple in Quantifiler HP, causing overestimated DNA concentrations. Purification of DNA extracts containing DTT is needed to overcome the effect. In general, inhibition of fluorescence detection should be considered as a possible restriction in qPCR. Careful review of fluorescence data is therefore recommended.

Conflict of interest

None.

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