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Improving qPCR Efficiency in Environmental Samples by Selective Removal of Humic Acids with DAX-8

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Abstract

Quantitative PCR is becoming the method of choice for the detection of pathogenic microorganisms and other targets in the environment. A major obstacle when amplifying DNA is the presence of inhibiting substances like humic acids that decrease the efficiency of PCR. We combined the polymeric adsorbent Supelite™ Dax-8 with a large-volume (10 mL) nucleic acid extraction method to decrease the humic acid content prior to qPCR quantification in water samples. The method was tested by spiking with humic acid standards and the bacterial surrogate *Acinetobacter baylyi* ADP1. Improvements in qPCR detection of ADP1 after application of Dax-8 resin (5 and 10 w/v %) were compared with the effects of added bovine serum albumin (BSA) (50, 100 and 200 ng/µL). Both additions improved detection of ADP1 by counteracting inhibitory effects. There were no changes in mean cycle threshold difference (ΔC_T) after application of DAX-8 compared to the control despite some loss of DNA, whereas significant increases occurred for BSA, irrespective of BSA concentration applied. The use of DAX-8 leads to an increase in qPCR amplification efficiency in contrast to BSA. The commonly used method to calculate genomic sample concentrations by comparing measured CT values relative to standard curves is only valid if amplification efficiencies of both are sufficiently similar. Dax-8 can provide this efficiency by removing humic acids permanently from nucleic acid extracts and has the potential to significantly increase the reliability of reported non-detects and measured results obtained by qPCR in environmental monitoring.

1. Introduction

Quantitative real-time PCR (qPCR) can be a sensitive and powerful diagnostic tool that is widely used in environmental research. Yet the use of qPCR-derived information on the abundance of microbial indicators or human pathogens for the development or implementation of water quality criteria is in its infancy, because of unresolved issues associated with the detection of low concentrations of microbial targets in natural waters. In particular, environmental samples may contain substances like phenolic compounds, heavy metals and humic acids that inhibit enzymatic reactions, which may lead to an underestimation of pathogen concentrations (Wilson, 1997). These inhibiting compounds are found in a variety of environmental samples and impact the sensitivity of both conventional and quantitative PCR (Johnson et al., 1995; Marlowe et al., 1997; Sluter et al., 1997; Tsai and Olson, 1992) (Audemard et al., 2004; Loge et al., 2002).

The serial dilution of samples provides an excellent means to overcome inhibiting effects when a gene target is present in high copy numbers (Queipo-Ortuno et al., 2008; Tsai and Olson, 1992). However, dilution can increase variation in measured gene copy numbers, particularly, when high dilutions are necessary (Kreader, 1996; Tsai and Olson, 1992). Furthermore, dilution can lead to false negatives when targets are diluted below their detection limit or when inhibitors cannot be diluted to levels below interference. Many protocols have been developed for the removal of inhibitors during nucleic acid extraction from samples with low target gene concentrations. Some of these methods include phenol–chloroform–isoamyl alcohol (25:24:1) extraction, which successfully

removed inhibitors from sediments (Tsai and Olson, 1991; Wiedbrauk et al., 1995). The addition of $AlNH_4(SO_4)_2$ during extraction also significantly reduced the co-purification of PCR inhibitors (Braid et al., 2003). Another approach involved embedding DNA in agarose gel and improved PCR amplification of templates in samples containing polysaccharides and humic acids (Moreira, 1998). A proposed multi step solution for solid environmental samples involved the precipitation of DNA with 5% polyethylene glycol 8000 (PEG) and 0.6M NaCl; filtration with a Sepharose 4B–polyvinylpolypyrrolidone (PVPP) spin column; and addition of skim milk (0.3% w/v) to the PCR reaction solution, which resulted in higher DNA purity albeit not improved detection limits (Arbeli and Fuentes, 2008).

A number of commercial extraction methods are useful for the purification of DNA or RNA from environmental waters, sediments, or sludges. Unfortunately, applicable sample volumes are usually limited and extraction efficiencies have been shown to vary from sample to sample or with matrix composition (Jiang et al., 2005).

Other approaches involve immunomagnetic separation (IMS) of cells and culture enrichment prior to DNA extraction to eliminate or reduce PCR inhibitors (Dudak and Boyaci, 2008; Fontaine and Guillot, 2003; Hibi et al., 2007; Lechevallier et al., 2000; Lowery et al., 2001). While useful, they are impractical for organisms for which there are no IMS procedures or which cannot be cultured. In addition, the use of IMS is expensive, and therefore is limited to using samples mostly for single organism detection.

In contrast, due to its convenient application as component in reaction mixtures bovine serum albumin (BSA) has been widely used to reduce inhibitory effects in PCR and qPCR assays of bacterial, protozoan, and viral gene targets (Al-Soud and Radstrom,

2000; Chandler et al., 1998; Forbes and Hicks, 1996; Jiang, et al., 2005; Kreader, 1996; Monpoeho et al., 2000; Poussier et al., 2002; Rochelle et al., 1997; Wang et al., 2007). The optimal BSA concentrations used varied from 40 ng/µL to 400 ng/µL. Studies reporting optimization of BSA concentrations for real-time PCR are rare. However, even though qPCR is supposed to be more sensitive than conventional PCR with lower detection limits, negative effects of BSA on qPCR have also been reported. For example improved amplicon detection at BSA concentrations greater than or equal to 100 ng/µL, gene copy numbers detected by qPCR were less, cycle threshold (C_T) values were increased, and melting temperature (T_m) values were altered for the real time PCR amplification of *hydA* genes of *Clostridium* spp. using degenerative and species-specific primers for *C. butyricum* (Wang et al., 2007). In another study it was observed that the addition of BSA or bovine lacto transfer technique optimizer (BLOTTO) to the PCR assay mixture in a multiplex qPCR using ß-giardin P241 (FAM-labeled) and COWP P702 (HEX-labeled) primers-probes, the efficiency of the amplification of the COWP gene in the multiplex qPCR was reduced from 100 to 70% (Guy et al., 2003).

With humic acids being the most prevalent inhibitors in natural surface waters, we tested DAX-8 as adsorbant to remove humic acids prior to qPCR. The non-ionic macroporous resin Supelite DAX-8 is known to reversibly adsorb humic substances that can then be further analyzed (McDonald et al., 2007; Peuravuori et al., 2002; Peuravuori et al., 2005). We compared qPCR performance of obtained extracts with and without DAX-8 treatment when four different BSA concentrations were added to the final qPCR mixture. The objective was to develop a protocol for sample pretreatment to reduce sample limits of detection (Rajal et al., 2007) and provide greater sensitivity and

reliability of qPCR assays needed to quantify microbial targets in natural waters and to assess (waste) water treatment performance.

2. Materials and Methods

2.1 Water sample preparation

Humic acid removal experiments were performed with two humic acid standards (HA-10: 10 mg/L and HA-90: 90 mg/L) and three concentrated surface water samples from an urban water body (MR-A, SC-B and SC-C). Stock solutions of humic acid were prepared by using solid humic acid mix (Fluka, no 53680, Sigma Aldrich, Inc, Milwaukee, WI, USA). These stock solutions were then diluted to give standard solutions of appropriate concentrations and spiked with *Acinetobacter baylyi* strain ADP1 (ATCC 33305) to a final concentration of 10^5 cells/mL for the evaluation of qPCR performance.

Three concentrated surface water samples (retentate) were obtained in October 2006 in the City of Stockton, California, processed with a hollow fiber ultrafiltration method, and extracted for nucleic acids prior to storage at -30°C. The filtration procedure is described in detail elsewhere (Rajal et al., 2007). The samples MR-A, SC-B and SC-C were concentrated from 100 L to 188 mL, 270 mL and 118 mL, respectively, after spiking with *Acinetobacter baylyi* ADP1 to a final concentration of 10^5 cells/mL prior to filtration. These three samples exhibited both the highest measured humic acid contents and most inhibited qPCR amplification from a set of 54 samples obtained during multiple stormwater sampling campaigns in California.

2.2 Nucleic acid extraction

To analyze a sufficiently large fraction of the original sample, 10 mL of the samples was added to a 200-mL conical plastic centrifuge bottle containing 40 mL of lysis buffer (Boom et al., 1990). The solution was vortexed for 15 seconds. After a 10-min incubation at room temperature, 40 mL of absolute ethanol was added to the lysed samples and vortexed for 15 seconds. The resultant lysate was spun in a centrifuge for 5 min at 4550 × g to pelletize solids. The entire supernatant was added to a QIAamp Maxi Spin column (Qiagen, Valencia, CA, USA) using a vacuum manifold (Qiagen) under a suction pressure of 800 mbar. The column was washed once with 5 mL buffer AW1 (Qiagen) and 5 mL buffer AW2 (Qiagen). The column was placed into a sterile 50-mL collection tube, centrifuged at 4,550 × g for 15 min, and incubated at 70°C for 10 min to remove traces of AW1 and AW2. Nucleic acid was eluted with an addition of 600 µL of ddH_2O, followed by centrifugation at 4550 × g for 5 min. Another 600 µL of ddH_2O was added to the column and centrifuged at 4550 × g for 10 min. Extracted nucleic acid was stored at -70°C until used for quantitative PCR analysis.

2.3 DNA concentration measurement with PicoGreen

The amount of DNA of *Acinetobacter baylyi* ADP1 (ATCC 33305) in the extracts was quantified using PicoGreen fluorescence (Ahn et al., 1996). A working solution of PicoGreen dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA) was prepared by diluting the concentrated dye solution 1:200 with TE Buffer in a plastic tube protected from light. The DNA samples were diluted with sterile nanopure water until an absorbance within the calibration range was achieved. Thirty microliter of the diluted

sample was mixed with 30 μL of the diluted PicoGreen reagent, followed by an incubation period of 3 min in the dark. The fluorescence was measured in triplicates with a TBS-380 Mini-Fluorometer (Turener Biosystems, Sunnyvale, CA) using a Minicell Adaptor. A standard curve was generated using Lambda phage DNA with concentrations between 0 and 100 ng/mL (Fluorescence = 0.9848 x (DNA concentration (ng/mL)) + 0.5162; $R^2 = 0.9999$).

2.4 Quantitative PCR of *Acinetobacter baylyi* ADP1 and Inhibition Factor

Each 25 μL PCR reaction volume contained 12.5 μL of commercially available TaqMan PCR mastermix (Eurogentec) with 400 nM each of forward and reverse primers and 80 nM probe for the respective TaqMan system dissolved in nuclease free water. For all qPCR reactions, 10 μL of the diluted gDNA sample was assayed in a final reaction volume of 25 μL. Cycling conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C, using an ABI Prism 7000 (Applied Biosystems). The sequences for primers and probes are Acinet-137F 5'-GATGCAACGCGAAGAACCTTA-3', 5'-TTCCCGAAGGCACCAATC-3', and 5'-6-FAM-CTGGCCTTGACATAGTAGAAACTTTC C-TAMRA-3' (Schriewer et al., 2010).

Duplicate nucleic acid extracts were quantified by qPCR and each replicate underwent five serial dilutions (undiluted, 1:3, 1:9, 1:27, 1:81 and 1:243). When C_T values were plotted against the logarithms of dilutions, data points within a linear range were determined to be without inhibition. The lowest dilution within this linear range was designated as inhibition factor. For example, if the dilutions 1:9, 1:27, 1:81, and 1:243 would mark the linear range then a inhibition factor of 9 would be recorded (Rajal et al., 2007).

2.5 Humic acid concentration measurements

The humic acid concentration in feed and retentate samples was measured by the method described elsewhere (Howeler et al., 2003). Stock solutions of humic acid were prepared by using solid humic acid mix (Fluka, no 53680, Sigma Aldrich, Inc, Milwaukee, WI, USA). These stock solutions were then diluted to give standard solutions of appropriate concentrations. A standard curve was established for the range of 0 to 100 mg/L (Absorbance = 0.0117 x (humic acid concentration (mg/L)) – 0.008; R^2 = 0.9989).

2.6 Inhibitor treatment

2.6.1 DAX-8

Supelite™ DAX-8 resin (Supelco, Bellefonte, PA), an insoluble polymer, was applied to the conical poly-ethylene centrifuge bottles after mixing sample and lysis buffer followed by a 10-min incubation period.

2.6.2 Bovine serum albumin (BSA)

BSA stock solution was prepared from crystallized BSA (Sigma-Alrich, St.Louis, MO) with DNA/RNAase free ddH_2O and added instead of nuclease-free water into the qPCR reaction mixture of 25 µL, to achieve final concentrations of 50, 100 and 200 ng/µL.

2.7 Amplification Efficiency Determination

For the determination of amplification efficiency used the window of log-linearity in the fluorescence signal of single amplification curves. Ramaker et al. (2003) used an iterative algorithm to search for lines consisting of at least four and no more than six data points with the highest R^2 value and a slope close to the maximum slope to eventually obtain the maximum efficiency for each individual amplification curve. The qPCR system (Applied Biosystems 7000 Sequence Detection System) we used creates one data point for every cycle and, unfortunately, within our dataset less than 10% of amplifications showed more than four cycles with log-linear amplification. For that reason we calculated the slopes between each two cycles and used the maximum slope when the next adjacent slope was within 10% deviation (Fig. 1). Accordingly, we were using a frame of four log-linear cycles or data points. Real-time PCR efficiencies (E) were calculated from the given lopes, according to the equation: E = 10(-1/slope) - 1, where E = 1 corresponds to 100% efficiency.

2.8 Statistical analysis

Standard statistical analyses were performed using MINITAB® version 15 (Minitab Inc., USA). All standard deviations were calculated from three independent replicates. The main and interaction effects of the variables tested were obtained after analysis of variance.

3 Results

3.1. Humic acid content and inhibition factors

Inhibition of qPCR can be expressed as the lowest uninhibited dilution of serial sample dilutions (Rajal et al., 2007). A plot of C_T value versus log dilution factor shows a linear relationship for points of uninhibited amplification. The required dilution step for a water sample represents the inhibition factor (Rajal et al., 2007).

For the measured concentrated water samples these inhibition factors were either 9 (MR-2D) or 27 (SC-3D and SC-3R) (Table 1). The inhibition factor for the 10 mg/L humic acid standard (HA-10) was 9 and no amplification at all could be observed for the 90 mg/L standard (HA-90). Consequently, a false negative result would occur if HA-90 represented an environmental sample.

Note that in real samples functional groups of humic acids may already be blocked by other water constituents, like ions or charged colloids, resulting in a lower inhibitory capacity compared to standard solutions. Hence, the observed inhibition effect with HA standards represents a worst-case scenario. Concentrated water samples had HA concentrations of 918.0 mg/L (SC-C), 1012.1 mg/L (MR-A), and 1198.7 mg/L (SC-B). Accordingly, the unfiltered samples had HA concentrations of 73.3 (MR-A), 96.8 (SC-C), and 106.2 mg/L (SC-B).

3.2 Inhibition Factors

DAX-8 was applied to samples or humic acid standards during nucleic acid extraction either at 5 or 10% (w/v). A significant decrease in qPCR inhibition was observed for all obtained nucleic acid extracts at both levels of Dax-8 (Table 1). DAX-8 relieved inhibition of PCR in one dilution for samples HA-10 and MR-A and in two dilutions for samples SC-B and SC-C. Remarkably, both levels of applied DAX-8 could counteract

inhibitory effects of 90 mg/L HA and allow amplification of *A. baylyi* ADP1. The lower DAX-8 concentration of 5% (w/v) was three times more effective than was 10% DAX-8. Overall, for every sample the use of DAX-8 resulted in reduced inhibition and, therefore, higher qPCR sensitivity.

BSA was added to the qPCR reaction mixture to obtain final concentrations of 50, 100 and 200 ng/µL. For all samples except HA-90 the result was identical to the DAX-8 treatment. Only a three-fold dilution was necessary to overcome inhibition. For the 90 mg/L humic acid standard it was possible to achieve detection within a linear range by diluting the original sample nine times when using 50 or 100 ng/µL or even only three times when using 200 ng/µL (Table 1).

3.4 Cycle threshold values

Paired t-tests comparing C_T values of untreated samples and samples treated with either DAX-8, BSA or combinations of both showed significant improvement only for treatments that had received BSA (Table 2). Significance levels were below 0.001 for a BSA concentration of 50 ng/µL (t: 4.54) and 200 ng/ µL (t: 4.50) and at 0.005 for a BSA concentration of 100 ng/µL (t: 3.25). All other combinations exhibited higher p-values. The lack of a statistically significant effect on C_T values for DAX-8 treated samples is remarkable, because qPCR performance was clearly improved as shown for inhibition factors and supported by the fact that target signals could be obtained in cases where untreated samples could not be amplified. The reason behind this discrepancy can be found in the loss of DNA during DAX-8 treatment. When DNA recoveries as assessed by pico green analysis of spiked nanopure water samples were compared only 35.6% (±

0.03) of *Acinetobacter* DNA could be recovered after nucleic acid extraction with 5% DAX-8 and only 28.8% (± 0.02) when using 10 % DAX-8 relative to untreated samples. The magnitude of DNA loss was proportional to the amount of applied DAX-8. Despite the loss of DNA no statistically significant variation in C_T values of the untreated samples could be observed, which was hypothesized to be due to the much improved qPCR efficiency.

We performed a simple additional experiment with nanopure water that was spiked with *Acinetobacter*. Here the DNA recovery could be improved to yield 67.7% ± 6.00% compared to the untreated extract. This time 10% (w/v) DAX-8 was applied before the extraction and removed by centrifugation, the resulting pellet was washed with a solution of 0.05 M Glycine containing 1% (v/v) Tween 80, and combined supernatants were then extracted the same way as for the other samples.

3.5 Amplification Efficiency

We studied the window of log-linearity in the fluorescence signal of single amplification curves to assess amplification efficiencies (see section 2.7.). Since not all of the data were normally distributed, the nonparametric Mann-Whitney test (also called Wilcoxon-Mann-Whitney (WMW)) was used to test for significant differences between qPCR efficiencies of untreated and treated samples (Table 3). Remarkably, every combination of 5% DAX-8 showed a statistically significant increase in qPCR efficiency, whereas none of the BSA additions alone had an effect. When 10% DAX-8 was applied 50% of the combinations with BSA still led to an increase in qPCR efficiency (Table 3).

4. Discussion

Quantitative PCR is a very powerful tool with an increasing number of publications in environmental sciences reporting quantitative PCR data. Inhibition of amplification particularly in environmental samples is a recognized problem. Most real time PCR data analysis procedures assume that PCR efficiencies of target amplifications are comparable to the efficiency of amplification obtained from pure standards. For example, it is reported that the comparative C_T method leads to a 4-fold error when PCR efficiencies vary only over a range of 0.04 (Ramakers et al., 2003). However, when working with environmental samples with inhibited amplification of target amplicon this range is far exceeded. As a direct consequence target amplifications should ideally exhibit the same amplification efficiency as that used to create a standard curve. Consequently, agents that remove or block inhibitory substances should not only increase the sensitivity of the PCR reaction but also guaranty ideal amplification rates. Whereas C_T values or mean measured gene copies have been frequently used to statistically assess differences in treatment methods, amplification efficiencies are only rarely mentioned.

Many studies discuss the impact of non-ideal amplification efficiencies on quantification and propose mainly mathematical solutions (Bar and Muszta, 2005; Liu and Saint, 2002; Nogva and Rudi, 2004; Pfaffl, 2001; Ramakers, et al., 2003). However, the use of amplification efficiencies of qPCR as criterion for the selection of methods is scarce, yet crucial for a sound evaluation (Gobbers et al., 1997; Sikorsky et al., 2004; Tichopad et al., 2002; Wolffs et al., 2004). Therefore, the aim of this study was to provide a new method for the selective removal of humic acids during nucleic acid

extraction and to compare the results with BSA as a widely accepted means for the improvement of qPCR performance (Duncan et al., 2006; Harmon et al., 2007; Pietarinen et al., 2008; Reischer et al., 2006).

Main advantages of quantitative real time PCR over conventional PCR are the increased sensitivity and the ability to produce quantitative information. As shown for the humic acid standard of 90 mg/L, inhibition can cause failure even of simple presence/absence determination. Therefore, both DAX-8 and BSA treatments are very valuable for improving the detection limits in the presence of humic acids at all applied levels of treatment. While all tested BSA levels can lower C_T-values significantly, the loss of DNA during extraction caused by DAX-8 treatment results in unchanged C_T-values.

The equation $N_c = N_0 \times (1+E)^c$ simply implies 100% amplification efficiency if each cycle the number of target sequences is doubled by DNA polymerase enzymes. This equation can be a bad representation of reality if PCR inhibitors like humic acids are present (Fig. 2). It has been suggested that humic acids inhibit amplification by binding to the polymerase, target DNA or co-factor magnesium ions (Roose-Amsaleg et al., 2001; Wilson, 1997). BSA apparently enables free coordination of all three components. This might be particularly effective at the beginning of the PCR when diffusion and access of abundant polymerase and magnesium ions to the low number of target DNA pieces is the rate limiting factor. Later on in the PCR when the number of targets to be amplified increases, either BSA might suffer some loss from degradation due to multiple temperature cycles or the bulky humic acid molecules might lead to simple steric inhibition; in either case the “boosting” effect of BSA seems to wear off. Since no effect

of different BSA concentrations on C_T levels could be observed in our study we assume the latter may happen. When humic acid molecules are totally removed from the reaction the effect of increased amplification efficiencies persists longer.

Whereas the use of DAX-8 to improve qPCR quantification is not described elsewhere, literature about the beneficial use of BSA is abundant (Wang et al., 2007). In good agreement with our results is the observed shift in melting temperatures with certain amounts of BSA (Wang et al., 2007). This shift would be perceivable after several cycles leading to non-optimal amplification rates.

In this study the hypothesis of no variance between different amounts of applied BSA could be confirmed by paired t-tests for each pair of used BSA concentration in the range from 50 to 200 ng/µL (data not shown). Depending on the amount of inhibitors and the matrix composition the optimum range of applied BSA seems to vary. This range should be optimized for each combination of primer/probe/target and sample matrix to achieve best conditions. The selective removal of humic acids by DAX-8 could be applied within or prior to any nucleic acid extraction method and does therefore not influence the complex qPCR system. The loss of DNA that comes with this purification method can be further minimized as shown. An optimal solution could be the use of DAX-8 as stationary filter medium. Dax-8 membrane filters were not available for this study and this approach has to be further tested. Since the effect of DAX-8 treatment dominates the outcome when combined with BSA the combination of both doesn't result in any further benefit.

In conclusion, both tested treatments DAX-8 and BSA resulted in increased detection by lowering inhibition effects of humic acids. The combination of DAX-8 and BSA does

not result in an additional benefit. The use of BSA increases qPCR performance in the initial phase so that the exponential phase is reached sooner than without BSA, while no effect of BSA could be observed in the range of 50 to 200 ng/µL. Lastly, the use of DAX-8 leads to an increase in qPCR amplification efficiency in contrast to BSA. The commonly used method to calculate genomic sample concentrations by comparing measured CT values relative to standard curves is only valid if amplification efficiencies of both are sufficiently close. Dax-8 can provide this effect by removing humic acids permanently from nucleic acid extracts. Its use has the potential to significantly increase the reliability of reported non-detects and measured results obtained by qPCR in environmental monitoring.

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FIGURE LEGENDS:

Fig. 1. Illustration for the determination of the slope for a considered log-linear range (solid circles) within an amplification curve. The slope between C_T values 17 – 18 (solid black) is the highest of any two points of the amplification curve with the condition that the slope between each of these two points and the next adjacent points (solid grey) varies by no more than 10%

Fig. 2. Example for the effect of inhibitors on PCR amplification efficiency. Field blank samples with added humic acid as inhibitors (dotted) and without inhibitors (solid line)

Table 1**.** Humic acid concentrations and inhibition factors of water samples and humic acid standards treated with Dax-8 or BSA

Treatment	HA-10[a]	HA-90[b]	MR-A[c]	SC-B[c]	SC-C[c]
	Inhibition factor				
No treatment	9	>81	9	27	27
Dax-8					
5% (w/v)	3	9	3	3	3
10% (w/v)	3	27	3	3	3
BSA					
50 ng/µL	3	9	3	3	3
100 ng/µL	3	9	3	3	3
200 ng/µL	3	3	3	3	3
Humic acid concentration (mg/L)[d]	10.0	90.0	1012.1	1198.7	918.0

[a] 10 mg/L humic acid standard, [b] 90 mg/L humic acid standard, [c] water samples,

[d] RSD: below 0.01 %

Table 2. Average cycle threshold differences (ΔC_T) between no-treatment and Dax-8 and BSA treatment and their statistical significance

Dax (w/v %)	BSA (ng/μL)	N	Δ C_T	SD[a]	SEM[b]	t[c]	P[d]
5	0	18	0.636	1.563	0.368	1.73	0.103
10	0	18	-0.428	1.693	0.399	-1.07	0.299
0	50	18	1.560	1.457	0.343	4.54	<0.001
5	50	18	0.528	1.466	0.346	1.53	0.145
10	50	18	-0.306	1.661	0.392	-0.78	0.445
0	100	18	1.001	1.306	0.308	3.25	0.005
5	100	18	0.592	1.455	0.343	1.73	0.103
10	100	18	-0.446	1.580	0.373	-1.20	0.248
0	200	18	1.575	1.486	0.350	4.50	<0.001
5	200	13	0.827	1.543	0.428	4.50	0.077
10	200	13	0.812	1.495	0.415	1.96	0.074

[a] Standard deviation

[b] Standard error of the mean

[c] The statistic used to test the hypothesis that the variance of a factor is equal to zero

[d] *P*-value is the smallest level of significance that would lead to rejection of the null hypothesis with the given data. We chose the common α-level of 0.05 to determine an acceptable level of significance

Table 3. Effect of DAX-8 and BSA on qPCR efficiency. The Mann-Whitney Test was used to compare Dax-8 and BSA treatments with controls.

Treatment		Effect	Statistical parameters			
DAX-8 w/v %	BSA ng/μL	Median qPCR efficiency	PE[a]	W[b]	P[c]	N
0	0	0.5828				23
5	0	0.7359	-0.1793	451.0	0.0056	28
10	0	0.6810	-0.1092	524.0	0.1173	29
0	50	0.6691	-0.0851	571.0	0.3744	30
5	50	0.8385	-0.2428	404.0	0.0001	30
10	50	0.8506	-0.2346	406.0	0.0001	30
0	100	0.5946	-0.0208	596.0	0.6602	30
5	100	0.7528	-0.1410	500.0	0.0306	30
10	100	0.6274	-0.0828	543.0	0.1643	30
0	200	0.5792	-0.0384	599.0	0.6996	30
5	200	0.7986	-0.1952	407.0	0.0021	24
10	200	0.8111	-0.2218	388.0	0.0005	24

[a] Point estimate, confidence level 95%

[b] Test statistic W is the sum of the m ranks assigned to the symbols x

[c] *P*-value is the smallest level of significance that would lead to rejection of the null hypothesis (no variance) with the given data. We chose the common α-level of 0.05 to determine an acceptable level of significance.

Fig. 1:

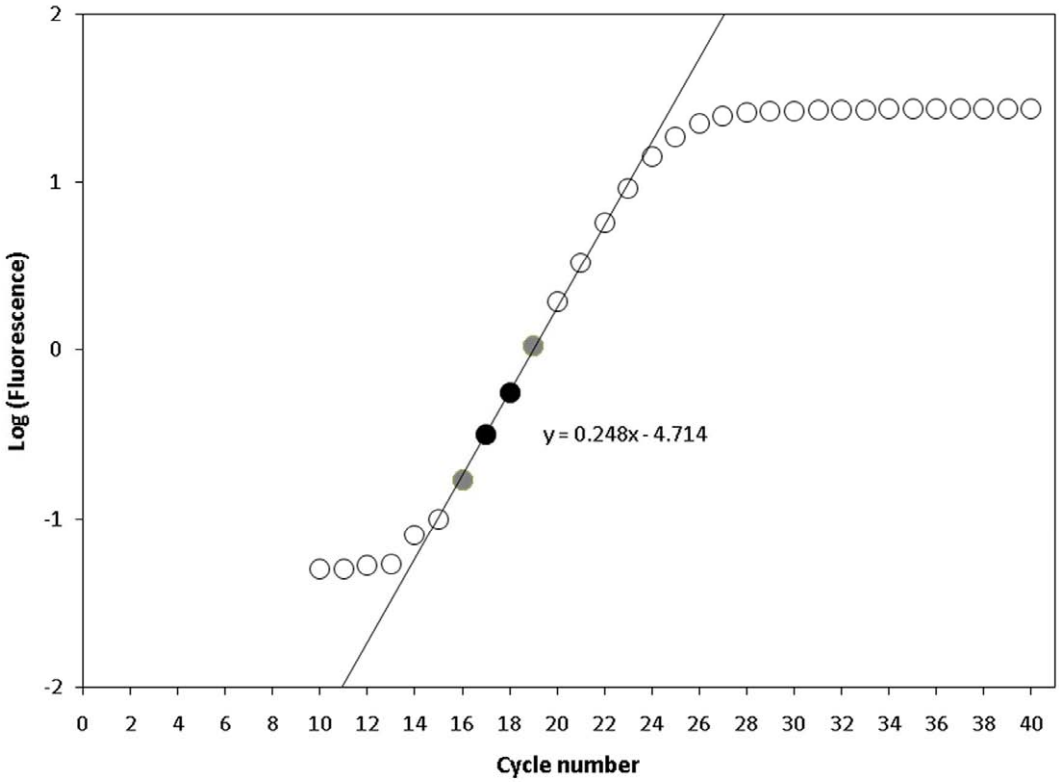
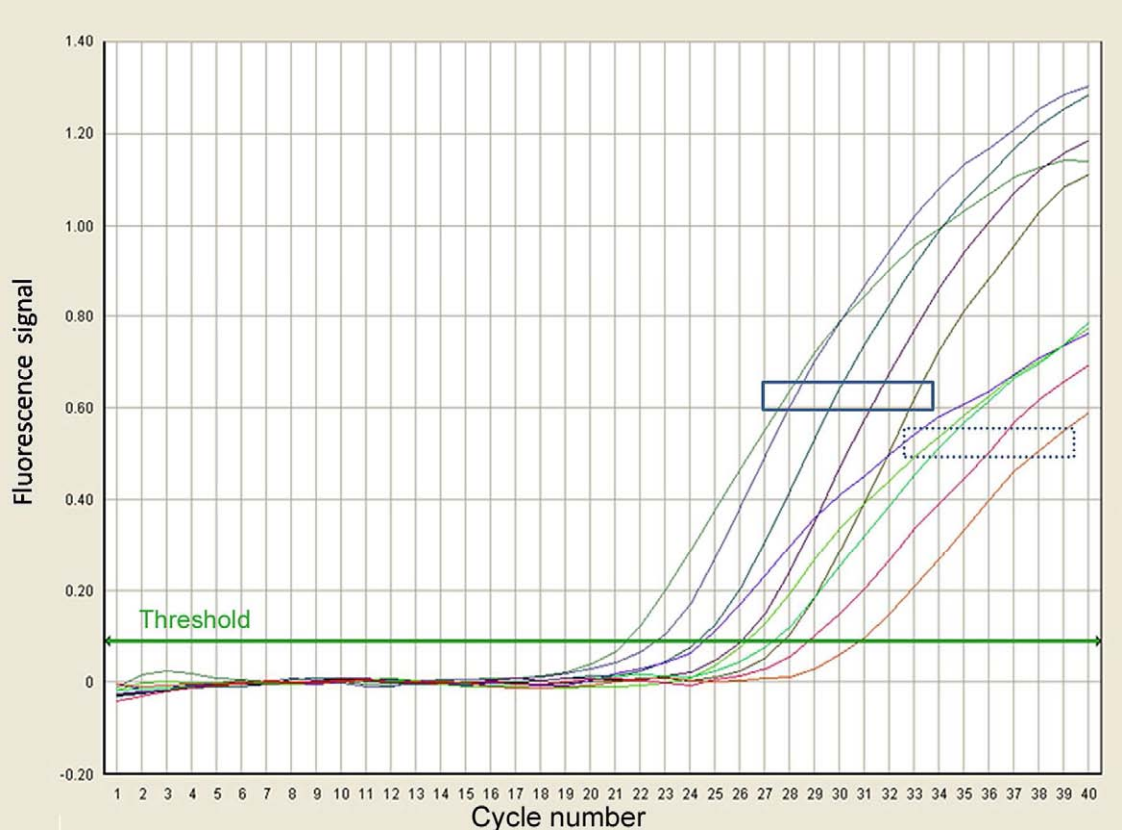


Fig. 2



Comment: Fig 2 color for web, black and white for print.