Preserving the integrity of the quantitative PCR workflow with Low DNA Binding Snap Cap Microcentrifuge Tubes

Introduction

Microcentrifuge tubes (MCTs) are often thought of as inert vessels that have little impact on experimental outcomes, but this is a misconception. They may contain leachable contaminants or cause nucleic acids to adhere to their surfaces, either of which can introduce error throughout the quantitative PCR (qPCR) workflow.

The polymer resins used to manufacture MCTs frequently contain plasticizers, solubilizing agents, and slip/release agents that are added to facilitate processing [1]. They may also contain stabilizers and biocides that make the finished products more resistant to degradation and microbial growth. Unfortunately, some of these additives can leach out of the plastic and contaminate samples during routine use.

Heating at temperatures above 37°C and contact with common nucleic acid extraction reagents have been shown to induce leaching of molecules that absorb at 220 nm and 260 nm from commercially available MCTs [2]. DNA absorbs ultraviolet (UV) light strongly at 260 nm. If a UV-absorbing substance leaches into a DNA sample, it can make the concentration in a spectrophotometric measurement appear considerably higher than it actually is. If the sample is used to prepare qPCR standards, the concentrations in the standards will be overestimated, making accurate quantitation impossible.

Reliable qPCR standards are critical for accurate quantitation and interpretation of amplification efficiency, but standards prepared in MCTs that adhere (bind) to DNA cannot be trusted. Adhesion is particularly problematic at low concentrations, where there may be as few as 10 copies of target DNA per reaction. Loss due to adhesion cannot be ignored at such low concentrations, because the error will be propagated in every qPCR cycle. Amplification efficiency will also appear lower than it is if the effective DNA concentration in the standards is reduced by adhesion.

To be confident in your results, you need a consistent supply of high-quality MCTs with minimal risk of contamination from leachable additives or error due to adhesion. This application note describes a performance evaluation of Low DNA Binding Snap Cap Microcentrifuge Tubes. The MCTs were assessed for qPCR inhibition, the presence of UV-absorbing leachable contaminants, and adhesion to human DNA. The results demonstrate that Low DNA Binding Snap Cap MCTs can be considered free of leachable PCR inhibitors and UV-absorbing contaminants. They can be used for short-term storage of DNA samples without compromising qPCR accuracy or amplification efficiency, and they consistently perform as well as or better than other low-binding MCTs on the market.

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Thermo Scientific™ Low DNA Binding Snap Cap Microcentrifuge Tubes and Thermo Scientific™ Low DNA Binding Snap Cap Microcentrifuge Tubes, Sustain™ Series were developed to preserve nucleic acid integrity for research, pharmaceutical, and forensics applications.

Materials and methods

PCR inhibition test

Low DNA Binding Snap Cap MCTs (1.5 mL, n = 11) and 1.5 mL MCTs from six other suppliers were tested for the presence of leachable PCR inhibitors by a third-party laboratory. A 1 mL aliquot of nuclease-free water was transferred to each MCT using a fresh pipette tip for each transfer. The process was repeated with a known inhibitor-free MCT from Supplier E, which was used as a negative control. Two inhibitor-positive MCTs served as positive controls. The MCTs were incubated in a heating block for 60 minutes at 95°C, then removed and allowed to cool to room temperature.

A 10 ng/µL stock solution of genomic DNA (gDNA) isolated from *E. coli* (ATCC™ 11303) was used to prepare serial dilutions for qPCR as outlined in Table 1. Water incubated in the MCTs was used as the diluent across concentrations, and a separate dilution series was prepared with water from each MCT. A no-template control (NTC) was also prepared using water incubated in the negative control MCT.

Table 1. Preparation scheme for the bacterial gDNA dilution series.

gDNA added	Water incubated at 95°C (µL)	Final volume (µL)	gDNA dilution	gDNA concentration (ng/µL)
5 µL from 10 ng/µL stock	45	50	A*	1
5 µL from A	45	50	cDNA-1	0.1
5 µL from cDNA-1	45	50	cDNA-2	0.01
5 µL from cDNA-2	45	50	cDNA-3	0.001
0	100	100	NTC	0

* Solution A was prepared to facilitate preparation of the dilution series but not tested.

A qPCR reaction mix was prepared with commercially available master mix, PCR-grade water, and primers targeting a conserved region of the *E. coli* 16S rRNA gene. Individual reactions contained 20 µL of reaction mix and 5 µL of template DNA. Each concentration in each dilution series was run in triplicate with the thermal cycling parameters shown in Table 2.

Table 2. Thermal cycling parameters for qPCR to assess PCR inhibition.

Temperature	Time	Cycles
95°C	10 min	1
95°C	10 sec	40
55°C	30 sec	
72°C	1 min	

The average C_t was calculated at each concentration for each dilution series. For an MCT to be considered free of leachable PCR inhibitors, the difference between the average C_t of dilutions prepared in MCTs from the same supplier and the average C_t of dilutions prepared in negative control MCTs could be no greater than one at any concentration ($\Delta C_t \leq 1$).

UV-absorbing leachable contaminant test

Low DNA Binding Snap Cap MCTs (1.5 mL, n = 11) and 1.5 mL MCTs from six other suppliers were tested for the presence of UV-absorbing leachable contaminants. A 300 µL aliquot of HPLC-grade water was pipetted into a glass vial (blank) and each MCT, and the MCTs were incubated in a 65°C water bath for approximately 18 hours. The MCTs were then removed from the water bath and allowed to cool to room temperature.

Testing was performed with the Thermo Scientific™ NanoDrop™ One^C Spectrophotometer. The instrument was blanked with 1 µL aliquots of HPLC-grade water from the glass vial. Absorbance was measured at 220 nm, 230 nm, 260 nm, and 280 nm, and the mean absorbance and standard deviation (SD) were calculated at each wavelength. The limit of detection (LOD) was set to 3 x SD_{blank}, and the limit of quantitation (LOQ) was set to 10 x SD_{blank}. Water incubated in the MCTs was tested using the same procedure. For each MCT, five measurements were recorded at each wavelength and averaged. For an MCT to be considered free of UV-absorbing leachable contaminants, the mean absorbance had to be below the LOD at each wavelength.

DNA binding experiment

Applied Biosystems™ TaqMan™ Control Genomic DNA (Cat. No. 4312660) and nuclease-free water were used to prepare a human gDNA dilution series (Table 3). Each solution was vortexed for 1 minute before preparing the next dilution. A NTC was also prepared using only nuclease-free water.

Low DNA Binding Snap Cap 1.5 mL MCTs and 1.5 mL MCTs from the other suppliers were labeled with the concentration identifiers shown in Table 3. A test replicate consisted of four MCTs from the same supplier labeled DNA-0, DNA-1, DNA-2, and DNA-3. The MCTs were grouped into test replicates according to supplier in a storage rack, and 55 µL diluted gDNA was aliquoted into each MCT labeled with the corresponding concentration identifier. The MCTs were stored at –80°C for 24 hours, and the process was repeated on subsequent days for a total of three racks of MCTs. Nine replicates at minimum were prepared per supplier.

Table 3. Preparation scheme for the human gDNA dilution series.

Volume of gDNA added	Nuclease-free water (µL)	Final gDNA concentration (pg/µL)	Concentration identifier	gDNA copies per 9 µL*
5.5 µL from 10 ng/µL stock	1,494.5	36.7	DNA-3	100
150 µL from DNA-3	1,350	3.67	DNA-2	10
150 µL from DNA-2	1,350	0.37	DNA-1	1
0	1,350	0	DNA-0	0

* The dilution scheme was based on the number of gDNA copies per 9 µL, because 9 µL would be used to quantify the gDNA by qPCR the next day. One copy of human gDNA = 3.3 pg.

qPCR was performed to quantify any loss of gDNA due to adhesion to the MCTs during storage at –80°C. Aliquots were transferred from the MCTs to individual wells in 12-well strip tubes to facilitate multichannel pipetting for qPCR. A fresh gDNA dilution series was also prepared in a popular line of low-binding MCTs on the day of qPCR as outlined in Table 3. Aliquots from these tubes served as positive qPCR controls.

In a 15 mL conical tube, 3.60 mL Applied Biosystems™ TaqMan™ Universal Master Mix II, no UNG (Cat. No. 4440043) was mixed with 230 µL of an Applied Biosystems™ TaqMan™ single-tube human RNase P assay (Cat. No. 4316831). A qPCR plate was prepared by aliquoting 11 µL of this mixture into individual wells along with either 9 µL from an MCT that had been stored at –80°C or 9 µL freshly prepared gDNA. Reactions labeled DNA-1, DNA-2, or DNA-3 contained 1, 10, or 100 copies of gDNA, respectively (Table 3). qPCR was run on the Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System using the cycling parameters shown in Table 4.

Table 4. Thermal cycling parameters for qPCR to assess DNA adhesion.

Temperature	Time	Cycles
50°C	2 min	1
95°C	10 min	1
95°C	15 sec	40
60°C	1 min	

qPCR was performed on subsequent days with aliquots from MCTs in the other two racks stored overnight at –80°C. A fresh gDNA dilution series was prepared each day, and aliquots from those tubes served as positive controls. An average C_t was calculated at each gDNA concentration for MCTs from each supplier and plotted against $\log_{10}$ of the gDNA concentration. The LINEST function in Microsoft™ Excel™ software was used to obtain best fit line statistics for MCTs from each supplier, and qPCR amplification efficiency was calculated using Equation 1. Propagated error was also calculated for MCTs from each supplier by multiplying the respective amplification efficiency by ln(10) times the error in the slope.

Equation 1 $\text{Efficiency} = [10^{(-1/\text{slope})}] - 1$

Results

PCR inhibition test

Dilutions prepared with water incubated at 95°C in Low DNA Binding Snap Cap MCTs passed with average ΔC_t values of –0.1, –0.2, and 0.0 at 0.001 ng/µL, 0.01 ng/µL, and 0.1 ng/µL, respectively (Table 5). These results indicate that Low DNA Binding Snap Cap MCTs can be considered free of leachable PCR inhibitors. Dilutions prepared with water incubated in MCTs from three of the other suppliers also passed at each concentration. Dilutions prepared with water incubated in MCTs from Supplier B showed inhibition at all but the highest concentration, and all dilutions prepared with water incubated in MCTs from Supplier F showed inhibition. As expected, dilutions prepared with water incubated in the inhibitor-positive MCTs failed with average ΔC_t values ranging from 1.3 to 2.2.

Leachable UV-absorbing contaminant test

All Low DNA Binding Snap Cap MCTs passed with mean absorbance readings below the LODs at all wavelengths tested (Table 6). These results indicate that Low DNA Binding Snap Cap MCTs can be considered free of leachable UV-absorbing contaminants. MCTs from Supplier A and Supplier B were the only other MCTs that met the criteria for passing. MCTs from three of the other suppliers had mean absorbance readings well above the LOQs at 220 nm and 230 nm. This included MCTs from Supplier C, which also had mean absorbance readings above the LOQs at 260 nm and 280 nm.

Table 5. Results of the PCR inhibition test. Pass Fail

	ΔC_t		
***E. coli* gDNA concentration**	**0.001 ng/µL**	**0.01 ng/µL**	**0.1 ng/µL**
Low DNA Binding Snap Cap MCTs	–0.1	–0.2	0.0
Supplier A MCTs	0.0	0.1	0.0
Supplier B MCTs	3.2	2.1	0.5
Supplier C MCTs	0.0	0.0	–0.1
Supplier D MCTs	–0.4	–0.3	–0.8
Supplier F MCTs	1.7	1.4	1.3
Inhibitor-positive control A	1.7	2.0	1.9
Inhibitor-positive control B	2.2	1.7	1.3

Table 6. Results of the leachable UV-absorbing contaminant test. Pass* Fail**

	ΔC_t			
	220 nm	**230 nm**	**260 nm**	**280 nm**
LOD	0.056	0.052	0.040	0.037
LOQ	0.188	0.179	0.135	0.125
Low DNA Binding Snap Cap MCTs	<LOD	<LOD	<LOD	<LOD
Supplier A MCTs	<LOD	<LOD	<LOD	<LOD
Supplier B MCTs	<LOD	<LOD	<LOD	<LOD
Supplier C MCTs	0.330	0.198	0.278	0.142
Supplier D MCTs	0.286	0.290	<LOD	<LOD
Supplier E MCTs	0.236	0.212	<LOD	<LOD
Supplier F MCTs	0.175	<LOD	<LOD	<LOD

* No UV-absorbing contamination detected.

** Absorbance between the LOD and LOQ indicates trace contamination. Absorbance above the LOQ is quantifiable, which could impact downstream quantitative measurements.

DNA binding experiment

qPCR amplification efficiency ranged from 103% to ~115% across suppliers (Table 7). Amplification efficiency with aliquots stored in Low DNA Binding Snap Cap MCTs was in the middle of this range at 108.2% with an associated propagated error of 24.1%. Although comparable amplification efficiency was observed with aliquots stored in the MCTs from other suppliers, the propagated error varied widely from 1.1% to 59.2%. These results show that Low DNA Binding Snap Cap MCTs can be used for short-term cold storage of human gDNA qPCR standards down to one copy per reaction without significant error or loss of amplification efficiency due to adhesion.

Table 7. Results of qPCR assay to evaluate adhesion of gDNA to MCTs stored overnight at –80°C.

MCTs	Average C_t			R^2	Slope	qPCR efficiency	Propagated error
	DNA-1	DNA-2	DNA-3				
Positive control MCTs	37.2	33.8	30.7	0.9994	–3.25	103.3%	19.5%
Low DNA Binding Snap Cap MCTs	37.1	34.1	30.8	0.9991	–3.14	108.2%	24.1%
Supplier A MCTs	37.0	34.2	30.8	0.9954	–3.12	109.3%	53.2%
Supplier B MCTs	37.4	34.2	30.9	0.9999	–3.24	103.7%	6.8%
Supplier C MCTs	37.1	34.0	30.8	1	–3.17	106.9%	1.1%
Supplier D MCTs	37.0	34.1	30.8	0.9994	–3.12	109.0%	19.3%
Supplier E MCTs	37.2	34.1	30.8	0.9996	–3.21	105.0%	16.0%
Supplier F MCTs	36.9	34.3	30.9	0.9945	–3.02	114.5%	59.2%

Conclusion

The results of this study demonstrate that Low DNA Binding Snap Cap Microcentrifuge Tubes can be considered free of leachable UV-absorbing contaminants and PCR inhibitors that could interfere with quantitation. These low-binding MCTs can be used for short-term storage of DNA standards at –80°C with no significant impact on quantitative accuracy or qPCR amplification efficiency, even at very low DNA concentrations. The performance of Low DNA Binding Snap Cap MCTs can be trusted in every stage of the qPCR workflow, allowing you to have more confidence in your data.

References

1. Olivieri A, Degenhardt OS, McDonald GR et al. (2012) On the disruption of biochemical and biological assays by chemicals leaching from disposable laboratory plasticware. *Can J Physiol Parmacol* 90(6):697–703.
2. Lewis LK, Robson MH, Vecherkina Y et al. (2010) Interference with spectrophotometric analysis of nucleic acids and proteins by leaching of chemicals from plastic tubes. *BioTechniques* 48:297–302.

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