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Impact of Long-Term Storage on Stability of Standard DNA for Nucleic Acid-Based Methods▽†

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Real-time PCR is dependent upon a calibration function for quantification. While long-term storage of standards saves cost and time, solutions of DNA are prone to degradation. We present here the benchmark treatment for preservation of DNA standards, involving storage in 50% glycerol–double-distilled water, whereby a deviation of 0.2 threshold cycle (C_T) values resulted after 100 days of storage.

Detection of nucleic acid targets is an important tool in medical, biological, environmental, and food-related diagnostic applications (12). Among various methodical parameters, the issue of stable standards to ensure reliable quantifiable data is of prime importance. In real-time PCR, the threshold cycle (C_T) method is usually employed to obtain quantitative information based on comparison of the samples with external DNA standards. These DNA standards are measured, serially diluted, and then used for the calibration line. Storage of DNA standards would be desirable, as this production procedure is elaborate. In the past, many studies dealt with DNA degradation in the context of basic nucleic acid research, but few investigations covering practical applications in the context of molecular diagnostics were carried out (2, 5, 8, 20). Degradation of nucleic acids features in the storage of specimens, for example, cerebrospinal fluid and whole blood, and other samples, including various pathogens, and in forensic applications (1, 3, 6, 9, 10, 13, 16, 18, 21). In a pharmaceutical context, the stability of plasmids during storage has been investigated (7, 11, 15). All these studies have investigated storage by freezing based on rapid freeze and thaw cycles included in the experiments, but long-term storage has been neglected.

The aim of the present study was to investigate solutions for standard DNA storage in the context of molecular diagnostics, including long-term storage in the experimental design.

DNA from *Listeria monocytogenes* EGDe (ATCC-BAA-679) was used as the DNA quantification standard for real-time PCR. Bacteria were maintained at −80°C using the Microbank technology (Pro-Lab Diagnostics, Richmond Hill, Canada). All bacteria were grown overnight in tryptone soy broth with yeast extract (Oxoid, Hampshire, United Kingdom) at 37°C.

For preparation of genomic DNA, 1 ml of a pure overnight culture was subjected to DNA isolation using the NucleoSpin tissue kit (Macherey-Nagel, Düren, Germany).

Real-time PCR was carried out as described previously by targeting a 274-bp fragment of the *prfA* gene of *L. monocytogenes* (4, 17). Real-time PCR was performed in an Mx3000p thermocycler (Stratagene, La Jolla, CA), and the 25-μl volume contained either 5 μl of template DNA or, in the case of glycerol storage, 1 μl of template DNA and 4 μl of double-distilled water (ddH_2O).

Genomic DNA samples were stored in 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl; Invitrogen, Lofer, Austria); 50% glycerol (Merck, Darmstadt, Germany) in 1× PCR buffer; and 50% glycerol in ddH_2O. Aliquots of the different concentrations of DNA standards were stored for 100 days at 4, 0, and −20°C. Real-time PCR results for the standard dilutions containing 1.58×10^5 and 1.58×10^4 initial genomic *Listeria* DNA target copies have been integrated into high-copy-number standards (HCS). Standards containing 1.58×10^3 and 1.58×10^2 initial target copies have been integrated into low-copy-number standards (LCS). During the 100-day period, measurements were carried out on a weekly basis by performing real-time PCR in duplicate. Changes in C_T values during the 100-day test interval were expressed as ΔC_T. The median (ΔC_{T50}) is the time-based average value at day 50. The final C_T value (ΔC_{T100}) is the average C_T value at the end of the test interval. The particular plots for all tested DNA standard concentrations and storage conditions are presented and itemized in the supplemental material with slopes and standard errors.

After evaluation of the results for different temperatures, freezing at −20°C was found to provide the best storage conditions and caused the least shift in the resulting C_T values (ΔC_T) after real-time PCR. Nevertheless, this shift would lead to a significant deviation of the resulting sample values if such DNA standards were to be used as calibration standards. Storage in 50% glycerol and 1× PCR buffer also did not support durability of the genomic DNA. The results of these experiments for *L. monocytogenes* HCS (HCS_{List}) and LCS_{List} are presented in Table 1. The mean standard errors for HCS_{List} and LCS_{List} under all tested conditions were 0.09 and 0.08 C_T values, respectively.

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TABLE 1. Results of long-term storage of *L. monocytogenes* genomic DNA

Storage conditions	HCS		LCS		HCS		LCS	
	ΔC_{T50} (% RSD[a])	Change (*n*-fold) in concn. based on copy no.[c]	ΔC_{T50} (% RSD[a])	Change (*n*-fold) in concn. based on copy no.[c]	ΔC_{T100} (% RSD[a])	Change (*n*-fold) in concn. based on copy no.[c]	ΔC_{T100} (% RSD[a])	Change (*n*-fold) in concn. based on copy no.[c]
Cooling (4°C); storage in buffer[b]	2.5 (±4)	5.3	3.1 (±4)	7.8	2.9 (±3)	7.0	3.6 (±5)	10.7
Freezing (0°C); storage in buffer[b]	2.5 (±4)	5.3	5.1 (±2)	29.6	2.9 (±4)	7.0	6.1 (±3)	53.5
Freezing (−20°C); storage in buffer[b]	1.2 (±6)	2.3	1.7 (±6)	3.0	1.5 (±9)	2.8	2.0 (±2)	3.7
Storage in glycerol-buffer[b] at −20°C	1.9 (±7)	3.6	1.9 (±2)	3.5	2.3 (±6)	4.7	2.2 (±3)	4.3
Storage in glycerol-ddH_2O at −20°C	0.6 (±3)	1.5	0.2 (±2)	1.1	0.4 (±7)	1.3	0.1 (±5)	1.1

[a] RSD of the results derived from measurement tolerance of the Mx3000p thermocycler.
[b] Buffer composition: 50% glycerol in 1× PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4).
[c] Biasing factor: change in sample concentration by means of deviation of the initial copy number. Values shown are the resulting errors in the values for the calculated sample copy numbers caused by the shift of the C_T values for the underlying basis of calculation by means of the calibration standard due to DNA degradation.

For evaluation of the influence of buffer composition on long-term storage in glycerol, HCS_{List} and LCS_{List} were stored in 50% glycerol in ddH_2O and in 50% glycerol in 1× PCR buffer. A comparison of the ΔC_{T50} and ΔC_{T100} values showed average differences of 1.9 and 2.1 C_T values for HCS_{List} and LCS_{List}, respectively, for the different buffer compositions. These findings correspond to respective errors in sample values of 3.4-fold for HCS_{List} and 3.2-fold for LCS_{List}. The final (ΔC_{T100}) values achieved for storage in 50% glycerol in ddH_2O are lower than all values obtained during long-term cooling and freezing. Specifically, the ΔC_{T100} values for storage of HCS_{List} and LCS_{List} in 50% glycerol in 1× PCR buffer were 2.3 (standard deviation [SD], ±0.14; relative standard deviation [RSD], ±6%) and 2.2 (SD, ±0.06; RSD, ±3%). The ΔC_{T100} values for storage of HCS_{List} and LCS_{List} in 50% glycerol in ddH_2O were 0.4 (SD, ±0.03; RSD, ±7%) and 0.1 (SD, ±0.01; RSD, ±5%), respectively.

The results of these experiments were surprising, as storage in 50% glycerol and 1× PCR buffer did not enhance the stability of the DNA as expected (2) and the outcome of storage by freezing at −20°C also did not meet expectations. The resulting shift of C_T values after real-time PCR during 100 days of storage would lead to a 4-fold deviation and up to 2 log scales of deviation of quantification for all storage conditions and high and low standard concentrations, respectively. For practical routine application, these data suggest the proximate production of standards for real-time PCR. Nevertheless, the possibility of long-term storage of DNA standards is desired, as it theoretically offers reliable data from one source and is time saving, but it is not without controversy (12, 14). Schaudien et al. investigated the stability of short DNA fragments (144 to 203 bp) in a short-term, rapid freeze and thaw study (19). Based on the results, it was concluded that glycerol prevents DNA shearing by ice crystal formation.

In contrast, this work clearly demonstrates that the stability of the DNA is also dependent on buffer composition. Our data indicate that the use of glycerol in the storage solution necessitates the avoidance of DNA-degrading substances in the buffer composition. In summary, the data suggest optimal long-term storage of standard DNA in solutions containing 50% glycerol in ddH_2O for practical use in clinical and surveillance diagnostics, as well as for operations in basic science. This strategy may prevent shearing due to ice crystal formation, thereby eliminating DNA degradation as a cause for deviating C_T values' leading to false quantification in real-time PCR.

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