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Evaluation of two matrices for long-term, ambient storage of bacterial DNA

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Abstract

Background: Culture-independent molecular analyses allow researchers to identify diverse microorganisms. This approach requires microbiological DNA repositories. The standard for DNA storage is liquid nitrogen or ultra-low freezers. These use large amounts of space, are costly to operate, and could fail. Room temperature DNA storage is a viable alternative. In this study we investigated storage of bacterial DNA using two ambient storage matrices, Biomatrica DNAstable® Plus and GenTegra® DNA.

Methods: We created crude and clean DNA extracts from five *Streptococcus pneumoniae* isolates. Extracts were stored at −30°C (our usual DNA storage temperature), 25°C (within the range of temperatures recommended for the products), and 50°C (to simulate longer storage time). Samples were stored at −30°C with no product and dried at 25°C and 50°C with no product, Biomatrica DNAstable® Plus, or GenTegra® DNA. We analyzed the samples after 0, 1, 2, 4, 8, 16, 32, and 64 weeks using the Nanodrop 1000 to determine the amount of DNA in each aliquot and by qPCR for the *S. pneumoniae* genes *lytA* and *psaA*. Using a 50°C storage temperature, we simulated 362 weeks of 25°C storage.

Results: The average amount of DNA in aliquots stored with a stabilizing matrix was 103%-116% of the original amount added to the tubes. This is similar to samples stored at −30°C (average 102%-121%). With one exception, samples stored with a stabilizing matrix had no change in *lytA* or *psaA* Ct value over time (Ct range ≤ 2.9), similar to samples stored at −30°C (Ct range ≤ 3.0). Samples stored at 25°C with no stabilizing matrix had Ct ranges of 2.2 – 5.1.

Conclusion: DNAstable® Plus and GenTegra® DNA can protect dried bacterial DNA samples stored at room temperature with similar effectiveness as at −30°C. It is not effective to store bacterial DNA at room temperature without a stabilizing matrix.

Keywords

DNA banking; ambient storage; preservation

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Introduction

Biorepositories around the world need to store DNA for long periods of time. Currently, the gold standard for long-term storage of DNA samples is in liquid nitrogen (−196°C) or in ultra-low freezers with temperatures ranging from −20°C to −80°C. The systems required for this type of storage take up large amounts of space, are costly to operate, and are at risk of failure (1). A review published in 2007 estimated at that time the yearly global cost of maintaining frozen DNA samples probably exceeded $100 million (2).

Room temperature DNA storage is emerging as a viable alternative to frozen storage and recent publications have shown the effectiveness of some of the currently available commercial products (3–13). Two of these ambient storage products, Biomatrica DNAstable® Plus and GenTegra® DNA, use chemical compounds to protect DNA against oxidation and hydrolysis. In addition, when the DNA-chemical compound solution is dried, a physical barrier is created as the chemical compound forms a protective coating around the DNA.

The advent of culture-independent molecular analyses allows researchers to identify diverse microorganisms in an ecosystem. This type of work is undoubtedly leading to repositories of DNA from microbiological sources. Our laboratory's primary need for long-term DNA storage is for genetic analysis of viral and bacterial organisms causing infectious diseases. We currently have a large repository of frozen bacterial isolates as well as DNA extracted from them, thus we are in a position of evaluate the usefulness of room temperature storage of bacterial DNA. In this study we investigated two long-term, ambient DNA storage matrices, Biomatrica DNAstable® Plus and GenTegra® DNA for the storage of bacterial DNA, and compared them to our current method of DNA storage, frozen at −30°C.

Materials and Methods

Creation and storage of DNA extracts

We retrieved from −70°C storage five isolates of *Streptococcus pneumoniae* (*S. pneumoniae*) bacteria representing serotypes 3, 7F, 8, 15B, and 22F, subcultured them onto Trypticase soy agar with 5% sheep blood (Remel, Lenexa, KS), and incubated them overnight at 37°C with 5% CO_2. Using 1 μL loops, we then transferred bacterial cells to 3.0 mL nuclease-free water and vortexed the suspensions to ensure uniformity. We heated the suspensions at 100°C for 10 minutes and centrifuged them at 13,000 rpm for 5 minutes. We removed the supernatant containing the crude DNA, setting aside half and further cleaning up the other half using the QIAamp DNA Blood Mini Kit's purification from tissues protocol (Qiagen, Valencia, CA). This procedure allowed us to have two types of DNA preparations and from here forward we will refer to them as crude DNA and clean DNA, respectively.

Using a Nanodrop 1000 (Nanodrop, Wilmington, DE) we determined the amount of DNA in each of our five crude and clean DNA stocks. We dispensed ≤1.0 μg of each stock into 1.25mL Sarstedt polypropylene tubes with o-ring seals. To ensure this amount of DNA was in each tube, sample volumes differed for aliquots of each stock. After dispensing the DNA

stocks into the tubes, DNA was stored with DNAstable® Plus (Biomatrica, San Diego, CA), with GenTegra® DNA (Pleasanton, CA), or with no stabilizing matrix (Figure 1). We treated DNA stored with DNAstable® Plus or with GenTegra® DNA as outlined in their respective product inserts (14, 15). In brief, DNAstable® Plus was used at one part matrix to four parts DNA sample; 5 μL of GenTegra® DNA was added regardless of DNA sample volume. We used a room temperature vacuum centrifuge (Labconco CentiVap, Kansas City, MO) to dry the samples. We stored the tubes at three temperatures: −30°C (our laboratory's usual DNA storage temperature), 25°C (which is within the range of storage temperatures recommended by the product's manufacturers), and 50°C (to simulate longer storage times than allowed for by this study) (16) (Table 1). To protect the dried DNA from becoming damaged by humidity and/or fluorescent light, all tubes were stored in opaque boxes that were placed in darkened incubators in rooms with low humidity (≤35%).

Testing of DNA extracts

We analyzed the samples after 0, 1, 2, 4, 8, 16, 32, and 64 weeks in storage. Using a 50°C storage temperature, we were able to simulate 362 weeks of 25°C storage (Table 1). A new aliquot was tested at each time point. At the time of testing, we reconstituted DNA stored dried with nuclease-free water to the same volume as the original, incubated the tubes at room temperature for at least 15 minutes, and gently mixed by pipetting. We determined DNA concentration, and subsequently total DNA, using the NanoDrop 1000; we tested for the *S. pneumoniae* genes *lytA* and *psaA* using real-time PCR (qPCR) (17). For each qPCR assay, we used a common positive control and adjusted the assay threshold to within +/− 2 cycle threshold (Ct) values of a previously determined mean for that control.

Analysis

As we have no reason to believe DNA from different serotypes of *S. pneumoniae* will behave differently in storage, for analysis we combined data from the five isolates. This gave us a single value for each storage condition and time point. Information we received from the product's manufacturers indicated their products had only been tested on clean DNA (personal communication), so we considered the crude DNA and clean DNA preparations as different samples. For the DNA yield analysis, we compared the DNA yield at all time points with the amount of DNA added to the tubes at time 0 and calculated an average percentage recovery for each storage temperature and condition. For the qPCR analyses, we compared the Ct value range across all time periods for each storage temperature and condition. We considered a Ct range of ≤3.0 cycles (~1 $\log_{10}$) to be the same (18).

Results

DNA yield(μg) over time for *S. pneumoniae* crude and clean DNA extracts stored at room temperature or −30°C with and without a stabilizing matrix are shown in Table 2. Compared with the amount of DNA added to the tubes at time 0, the average percent recovery for samples stored with a stabilizing matrix ranged from 103%-116% which is similar to that found for samples stored at −30°C (102% and 121%). Samples stored at room temperature without a stabilizing matrix showed an average percent recovery of 98% and 116% for the

crude and clean preparations, respectively. The >100% recovery is likely due to variances in Nanodrop 1000 measurements.

qPCR testing for the *S. pneumoniae*-specific gene, *lytA*, showed that samples stored with a stabilizing matrix had no change in Ct values over time (Ct range ≤ 2.3 cycles), similar to samples stored in our usual manner, −30°C (Ct range = 2.0 cycles; Table 3). Clean preparation samples stored without a stabilizing matrix had increase in Ct value over time (Ct range = 3.2 cycles) but the crude preparation samples did not (Ct range = 2.2 cycles).

Real time PCR testing for the *S. pneumoniae*-specific gene, *psaA*, showed that, with the exception of the clean preparation samples stored with DNAstable® Plus (Ct range = 3.1 cycles), samples stored with a stabilizing matrix had no change in Ct values over time (Ct range ≤ 2.9 cycles; Table 4). This is similar to the Ct range for samples stored at −30°C (Ct range = 3.0 cycles). Both crude and clean preparation samples stored dried without a stabilizing matrix had a change in Ct value over time (Ct range ≥ 3.7 cycle).

Discussion

This study was designed to evaluate two DNA stabilizers, DNAstable® Plus and GenTegra® DNA, on the ambient storage of bacterial DNA extracted using crude and clean methodologies. As far as we know this is the first study that has investigated ambient temperature storage products on DNA from bacteria. This is an important addition to the literature since culture-independent technologies are leading to increased interest in microbiome studies. In turn, samples from these studies need to be stored as safety and efficiently as possible. Additionally, studies on DNA storage have not included data for DNA created from crude methodologies such as one of the ones we used.

Our evaluation took place over 64 weeks with treated samples stored at both 25°C and 50°C. High temperature stress allowed us to induce accelerated DNA aging according to the Arrhenius law (16). This gave us the ability to evaluate the products for the equivalent of nearly seven years. Our results show that both crude and clean preparations of bacterial DNA stored dried with either product maintain integrity for downstream PCR applications for this length of time.

We used two measures to evaluate the effectiveness of the aforementioned stabilization reagents: DNA yield and the Ct values from two qPCR assays that detect the *S. pneumoniae* genes *lytA* and *psaA* (17). These tests were chosen because they represent measures that will show whether or not the treated DNA would be of sufficient quantity and quality to be detected in our usual assays. Our need for ambient stability products will be to store DNA extracts from bacterial and viral organisms in high concentrations and for long periods of time for use in PCR, qPCR, and sequencing assays. When compared with time 0, DNA yield was similar over time for samples stored at room temperature indicating there was no loss in total DNA in the samples.

With one exception, samples stored dried with either stability product had no change in Ct value over time similar to samples stored in our usual manner, −30°C. The single exception gave a Ct range of 3.1 cycles, just over the cutoff for no difference. It is not clear why that

one group of samples would show a change, but it is unlikely due to a problem with the biostability product but rather an issue with the test process at one of the time points. As a control, we also dried samples, stored them at room temperature, but did not add a stabilizing matrix. These samples had Ct ranges that show this storage method does not adequately maintain DNA quality for downstream testing. Interestingly, clean preparation samples stored in this manner fared worse than crude preparation samples. Although it is unclear why this would be so, it is possible the crude preparation samples contained substances that, upon drying, partially protected the DNA. Those substances were likely removed in the process of creating the clean preparations.

This study has two limitations. The first is that it may not be generalizable to other applications that use stored DNA. Earlier we described our need for DNA stability products and how we typically use DNA extracts. The assays we use amplify short segments (100 – 500 bp) of single genes which we know are more likely to remain intact while longer segments may degrade. We did not test for overall DNA degradation which may be important to other laboratories storing and testing DNA from other sources or for other purposes. However, other studies have shown the usefulness of these two products using a variety of different testing parameters and starting material (7–13). We believe our data add to the literature and will be useful to laboratories who may be considering a transition to ambient storage protocols. Second, this study was originally intended as a nested study within a larger agency-wide study, thus we chose only five samples for testing in our laboratory. Unfortunately, issues beyond our control prevented the larger study from being completed and we are left with a small sample size. However, we believe the stability of the measures over time, even in this small sample size, is worthy of adding to the body of literature on this subject. Additionally, there are similar studies of biostability products that also have small sample sizes (8, 9, 12, 13, 19).

In summary, our data show DNAstable® Plus and GenTegra® DNA can protect both crude and clean preparations of dried bacterial DNA stored at room temperature with similar effectiveness as the same DNA samples stored at −30°C. Our accelerated aging experiments show that dried DNA can be preserved with these products for at least 6 years and 11 months.

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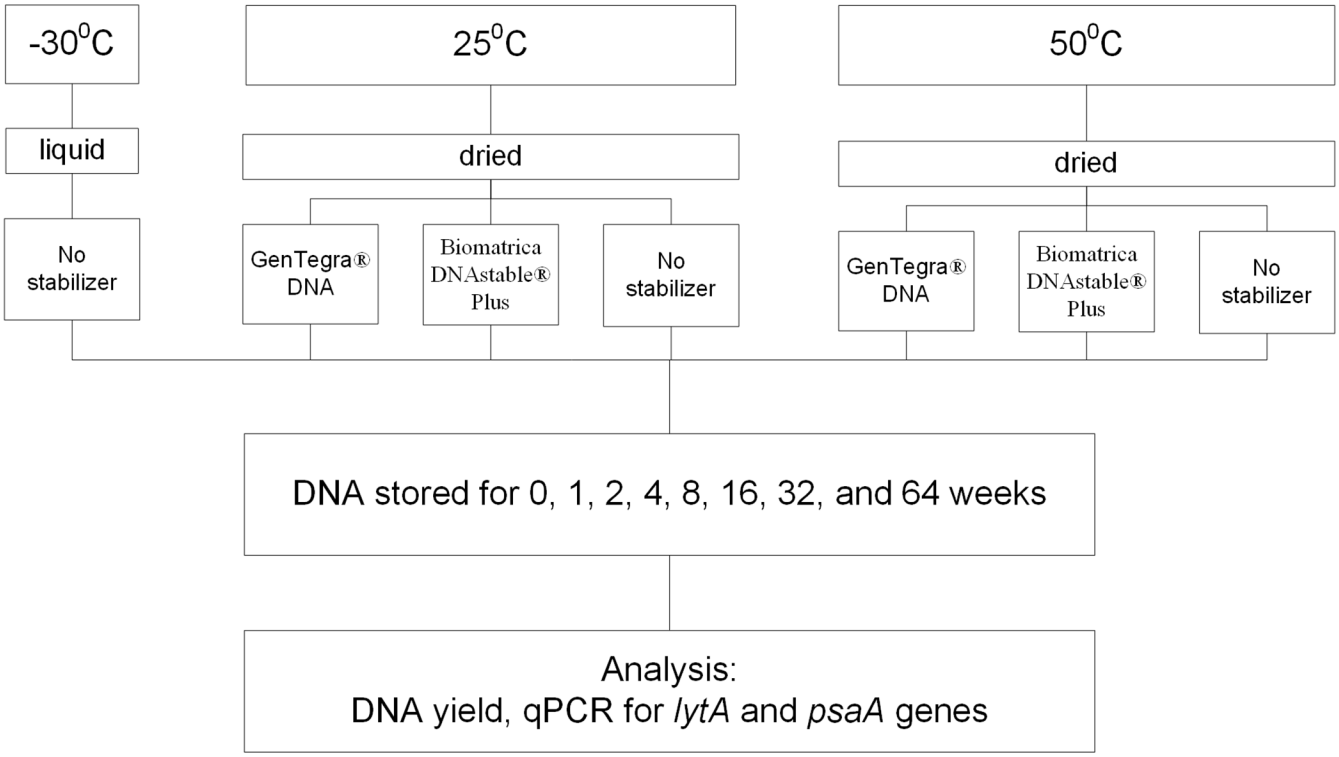

FIG. 1.
Specimen processing and testing workflow.

Table 1.

Room temperature equivalent of storage at 50°C.

Actual storage time at 50°C	Room temperature equivalent
1 week	5.6 weeks
2 weeks	11.3 weeks
4 weeks	22.6 weeks
8 weeks	45.2 weeks
16 weeks	90.5 weeks
32 weeks	180.9 weeks
64 weeks	362.0 weeks

Table 2.

Total DNA (μg) over time for *Streptococcus pneumoniae* DNA extracts stored at room temperature and −30°C.

Crude Preparation								
Temperature	**−30°C**		**Room Temperature**					
Stabilizer	**None**		**None**		**Gentegra® DNA**		**DNAStable® Plus**	
	μg (SD[b])	**Percent recovery[c]**	**μg (SD)**	**Percent recovery**	**μg (SD)**	**Percent recovery**	**μg (SD)**	**Percent recovery**
0 week	0.83 (0.04)	n/a	0.83 (0.04)	n/a	0.93 (0.04)	n/a	1.02 (0.11)	n/a
1 week	0.85 (0.05)	102	0.75 (0.13)	90	1.05 (0.23)	113	1.04 (0.05)	102
2 weeks	0.89 (0.05)	107	0.82 (0.11)	99	1.05 (0.05)	113	1.08 (0.05)	106
4 weeks	0.95 (0.06)	114	0.82 (0.18)	99	0.99 (0.06)	106	1.07 (0.09)	105
5.6 weeks[a]			0.82 (0.17)	99	1.00 (0.10)	108	1.08 (0.03)	106
8 weeks	1.16 (0.61)	140	0.82 (0.10)	99	0.95 (0.07)	102	0.97 (0.26)	95
11.3 weeks[a]			0.83 (0.16)	100	1.06 (0.05)	114	1.13 (0.05)	111
16 weeks	1.12 (0.33)	135	0.80 (0.11)	96	0.93 (0.11)	100	1.00 (0.11)	98
22.6 weeks[a]			0.82 (0.15)	99	0.99 (0.08)	106	1.06 (0.22)	104
32 weeks	1.12 (0.33)	135	0.91 (0.04)	110	1.14 (0.40)	123	1.29 (0.39)	126
45.2 weeks[a]			0.78 (0.14)	94	1.00 (0.04)	108	1.09 (0.07)	107
64 weeks	0.97 (0.25)	117	0.74 (0.17)	89	0.79 (0.44)	85	1.12 (0.06)	110
90.5 weeks[a]			0.80 (0.13)	96	0.99 (0.11)	106	1.01 (0.08)	99
180.9 weeks[a]			0.84 (0.10)	101	1.32 (0.41)	142	1.13 (0.07)	111
362.0 weeks[a]			0.70 (0.17)	84	0.96 (0.07)	103	1.12 (0.25)	110
Average		121		98		109		106
Clean Preparation								
Temperature	**−30°C**		**Room Temperature**					
Stabilizer	**None**		**None**		**Gentegra® DNA**		**DNAStable® Plus**	
	μg (SD[b])	**Percent recovery[c]**	**μg (SD)**	**Percent recovery**	**μg (SD)**	**Percent recovery**	**μg (SD)**	**Percent recovery**
0 week	0.58 (0.08)	n/a[d]	0.53 (0.21)	n/a	0.66 (0.17)	n/a	0.65 (0.13)	n/a

Crude Preparation								
Temperature	**−30°C**		**Room Temperature**					
Stabilizer	**None**		**None**		**Gentegra® DNA**		**DNAStable® Plus**	
	µg (SD[b])	**Percent recovery[c]**	**µg (SD)**	**Percent recovery**	**µg (SD)**	**Percent recovery**	**µg (SD)**	**Percent recovery**
1 week	0.52 (0.05)	90	0.67 (0.45)	126	0.73 (0.39)	111	0.57 (0.05)	88
2 weeks	0.56 (0.03)	97	0.51 (0.02)	96	0.74 (0.19)	112	0.69 (0.08)	106
4 weeks	0.64 (0.27)	110	0.51 (0.06)	96	0.66 (0.08)	100	0.71 (0.06)	109
5.6 weeks[a]			0.45 (0.15)	85	0.57 (0.07)	86	0.68 (0.10)	105
8 weeks	0.60 (0.23)	103	0.47 (0.06)	89	0.61 (0.04)	92	0.69 (0.12)	106
11.3 weeks[a]			0.60 (0.08)	113	0.62 (0.04)	94	0.75 (0.07)	115
16 weeks	0.45 (0.33)	78	0.71 (0.12)	134	0.78 (0.11)	118	0.92 (0.05)	142
22.6 weeks[a]			0.65 (0.04)	123	0.84 (0.50)	127	0.84 (0.29)	129
32 weeks	0.80 (1.25)	138	0.68 (0.14)	128	0.74 (0.17)	112	0.92 (0.47)	142
45.2 weeks[a]			0.70 (0.38)	132	0.61 (0.05)	92	0.66 (0.06)	102
64 weeks	0.56 (0.13)	97	0.52 (0.13)	98	0.53 (0.07)	80	0.75 (0.05)	115
90.5 weeks[a]			0.74 (0.03)	140	0.73 (0.11)	111	0.82 (0.12)	126
180.9 weeks[a]			0.74 (0.07)	140	0.83 (0.23)	126	0.93 (0.52)	143
362.0 weeks[a]			0.69 (0.09)	130	0.50 (0.03)	76	0.61 (0.09)	94
Average		102		116		103		116

[a] accelerated aging experiment

[b] standard deviation

[c] compared with time 0

[d] not applicable

Table 3.

Mean and standard deviation (SD) of *lytA* cycle threshold (Ct) over time for *Streptococcus pneumoniae* DNA extracts stored at room temperature and −30°C.

DNA	Crude Preparation				Clean Preparation			
Temperature	−30°C	Room Temperature			−30°C	Room Temperature		
Stabilizer	None Ct (SD)	None Ct (SD)	Gentegra® DNA Ct (SD)	DNAStable® Plus Ct (SD)	None Ct (SD)	None Ct (SD)	Gentegra® DNA Ct (SD)	DNAStable® Plus Ct (SD)
0 week	20.6 (0.68)	20.7 (1.13)	20.5 (0.81)	20.9 (0.66)	20.1 (0.64)	20.0 (0.89)	20.4 (1.07)	20.2 (1.08)
1 week	19.5 (1.48)	20.2 (0.88)	20.2 (0.76)	20.5 (0.57)	19.6 (0.55)	19.8 (0.54)	20.0 (0.65)	20.4 (0.64)
2 weeks	18.9 (1.32)	19.8 (0.70)	19.5 (0.63)	19.9 (0.61)	19.0 (0.52)	19.1 (0.46)	19.5 (0.77)	19.5 (0.80)
4 weeks	19.4 (1.27)	20.3 (0.86)	20.0 (0.66)	20.3 (0.53)	19.4 (0.55)	19.6 (0.74)	20.0 (0.60)	20.4 (0.55)
5.6 weeks[a]		19.9 (0.78)	20.4 (0.76)	20.5 (0.57)		19.7 (0.53)	20.0 (0.76)	20.5 (0.83)
8 weeks	19.8 (1.30)	20.4 (0.63)	20.5 (0.34)	20.5 (0.90)	19.8 (0.42)	20.5 (1.39)	20.4 (1.03)	20.1 (0.58)
11.3 weeks[a]		19.7 (0.73)	19.6 (0.69)	19.9 (0.60)		19.2 (0.61)	19.7 (0.81)	19.7 (0.70)
16 weeks	19.9 (1.19)	20.5 (0.66)	20.2 (0.82)	20.6 (0.77)	19.8 (0.55)	19.8 (0.48)	19.8 (0.60)	20.3 (0.81)
22.6 weeks[a]		20.3 (0.67)	20.1 (0.69)	20.6 (0.64)		20.0 (0.43)	19.8 (0.62)	20.3 (0.75)
32 weeks	20.9 (1.39)	21.8 (0.74)	21.6 (0.83)	22.0 (0.58)	21.1 (0.47)	21.0 (0.43)	21.1 (0.40)	21.7 (0.66)
45.2 weeks[a]		21.2 (0.75)	20.5 (0.75)	20.6 (0.76)		20.4 (0.92)	20.2 (0.85)	20.6 (0.54)
64 weeks	20.3 (1.50)	21.2 (0.76)	20.9 (0.70)	21.1 (0.72)	20.3 (0.94)	20.8 (0.67)	20.6 (0.52)	20.8 (0.55)
90.5 weeks[a]		20.7 (0.75)	20.3 (0.72)	20.6 (0.76)		20.6 (0.70)	19.9 (0.52)	20.3 (0.67)
180.9 weeks[a]		21.9 (0.72)	21.4 (0.59)	22.2 (0.74)		22.3 (0.46)	21.3 (0.50)	21.6 (0.91)
362.0 weeks[a]		21.6 (0.69)	20.9 (0.73)	21.3 (0.54)		22.2 (0.60)	20.6 (0.59)	21.1 (0.52)
Ct range[b]	2.0	2.2	2.1	2.3	2.1	3.2	1.8	2.2

[a] accelerated aging experiment

[b] widest range for the column; range of ≤3.0 cycles(~1 $\log_{10}$) is considered not different

Table 4.

Mean and standard deviation (SD) of *psaA* cycle threshold (Ct) over time for *Streptococcus pneumoniae* DNA extracts stored at room temperature and −30°C.

DNA	Crude Preparation				Clean Preparation			
Temperature	−30°C	Room Temperature			−30°C	Room Temperature		
Stabilizer	None Ct (SD)	None Ct (SD)	Gentegra® DNA Ct (SD)	DNAStable® Plus Ct (SD)	None Ct (SD)	None Ct (SD)	Gentegra® DNA Ct (SD)	DNAStable® Plus Ct (SD)
0 week	22.5 (0.95)	22.5 (1.43)	22.0 (1.18)	22.5 (0.98)	21.8 (0.94)	22.3 (0.91)	22.2 (0.97)	21.9 (0.99)
1 week	22.0 (1.80)	22.5 (1.50)	22.1 (1.09)	22.9 (0.82)	22.2 (0.80)	22.2 (0.94)	22.1 (1.11)	22.6 (1.02)
2 weeks	21.1 (1.73)	22.1 (1.09)	21.7 (0.96)	22.0 (0.90)	21.2 (0.96)	21.3 (0.92)	21.5 (1.38)	21.7 (1.28)
4 weeks	21.6 (1.59)	22.8 (1.11)	22.0 (0.81)	22.7 (0.83)	21.8 (0.96)	22.0 (1.10)	21.9 (1.26)	22.8 (0.93)
5.6 weeks[a]		22.0 (1.21)	22.3 (0.88)	22.8 (0.84)		22.2 (0.79)	22.2 (1.25)	22.8 (1.11)
8 weeks	21.6 (1.71)	22.3 (0.83)	22.2 (0.55)	22.2 (1.22)	21.6 (0.57)	22.0 (1.17)	22.1 (0.98)	21.9 (0.77)
11.3 weeks[a]		21.9 (1.07)	21.6 (0.94)	22.1 (0.93)		21.6 (0.96)	21.8 (1.29)	21.9 (1.13)
16 weeks	22.2 (1.60)	22.8 (1.12)	22.1 (1.01)	22.7 (1.04)	21.9 (1.03)	22.0 (0,88)	21.9 (0.99)	22.3 (1.15)
22.6 weeks[a]		22.9 (0.95)	22.3 (1.04)	22.7 (0.86)		22.7 (0.59)	22.1 (1.11)	22.7 (1.08)
32 weeks	21.9 (1.79)	22.7 (0.98)	22.1 (0.86)	22.7 (0.85)	22.0 (0.86)	22.0 (0.81)	21.8 (0.82)	22.4 (0.97)
45.2 weeks[a]		23.2 (0.88)	22.1 (0.99)	22.4 (1.02)		22.8 (1.32)	21.9 (0.90)	22.3 (0.74)
64 weeks	24.1 (2.17)	25.2 (1.45)	24.5 (1.07)	24.8 (1.24)	24.1 (1.86)	24.7 (1.27)	24.2 (0.95)	24.5 (0.97)
90.5 weeks[a]		22.9 (1.03)	22.3 (0.91)	22.6 (1.00)		23.6 (1.02)	21.8 (0.87)	22.4 (0.91)
180.9 weeks[a]		23.0 (1.03)	22.0 (0.88)	23.0 (0.97)		24.0 (1.01)	21.9 (0.80)	22.3 (1.21)
362.0 weeks[a]		25.6 (0.90)	24.4 (1.05)	24.9 (0.84)		26.4 (1.22)	24.2 (1.00)	24.8 (0.75)
Ct range[b]	3.0	3.7	2.9	2.9	2.9	5.1	2.7	3.1

[a] accelerated aging experiment

[b] widest range for the column; range of ≤3.0 cycles(~1 $\log_{10}$) is considered not different