APPLICATION NOTE

Plasmid DNA characterization for reliable pDNA purity and sizing for multiuser analysis.

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Introduction

Plasmid DNA (pDNA) are circular DNA used for vaccine development, monoclonal antibody production and gene therapies (1,2). When working with pDNA or pDNA-derived therapeutics, it is important to understand and monitor the effect of pDNA isoform composition on transfection efficacy, manufacturability and therapeutic function (3). Similarly, it is critical to be able to perform robust experiments for stability testing of pDNA molecules. Factors such as sample age and freeze thaw number may affect plasmid DNA purity. Degradation products may include linear and open-circular isoforms. Here we show a three user / three instrument study to measure a set of stressed plasmid DNA samples on the LabChip™ GXII Touch™ instrument using the LabChip Plasmid DNA Assay. The inter-run CV and stability results of a 6 kb pDNA standard are shown in this technical note.

Assay overview and experimental set-up

pDNA (pTXB1 plasmid, 6706 bp, New England BioLabs) was used as the test sample. The stock solution was diluted to 10 ng/µL using the LabChip Plasmid DNA Assay sample buffer and stored at 4°C. Aliquots were prepared and set aside for various stress conditions. Thermal stress used two ovens set at 40°C and 60°C. Freeze-thaw cycles were completed by transferring aliquots between -80°C and ~22°C at one-hour intervals. Once a stress condition was complete, the sample was stored at 4°C until testing.

Measurements of pDNA purity were obtained using a LabChip GXII Touch instrument (CLS138160) using the LabChip Plasmid DNA Assay (CLS160450) and the Plasmid DNA LabChip (CLS160538). Prior to measurement, samples were diluted to the recommended input concentration of 500 pg/µL using the LabChip pDNA Assay sample buffer. Data analysis was performed using the LabChip Reviewer software.

Three first-time assay users (in teams of 2 for this experiment) performed the test on three different LabChip GXII Touch instruments using three different chips. All testers used the same lot of reagents and same stock solutions of stressed pDNA samples. Each control or stressed sample was tested in duplicate in a given run.

Results and discussion

Measurement of pDNA control samples

Each group measured the pDNA control sample stored at 4°C. A representative electropherogram of the control sample is shown (Figure 1). The electropherogram shows a free dye, lower marker, supercoiled main peak (SC) and open-circular impurity (OC) (Figure 1). Sizing alignment was performed using the lower marker peak, which is a 300 bp DNA internal standard. A small fluorescent dye present in the marker solution is detected with migration time before the free dye. Whereas dye peaks can be used as lower marker references in many DNA LabChip assays, we found sizing performance is improved when using a DNA internal standard for the LabChip Plasmid DNA Assay. The average percentage purity of the control sample measurements was 88.31% ± 2.39 for the three analysts. The inter-run CV for the method was 2.71% for the supercoiled main peak. Based on the low percent average CV less than 5%, the control sips provide a reference to compare the stressed samples. The average size of the SC isoform for all measurements (N6) was 6903 ± 500 bp corresponding to accuracy of 3%.

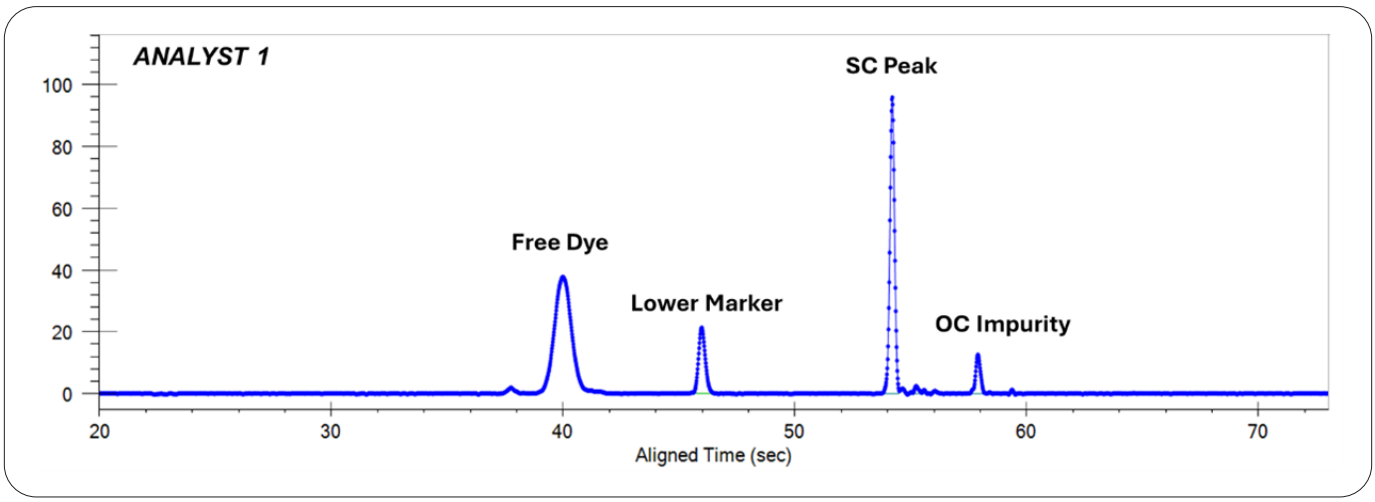

Figure 1: Example electropherogram of PTXB1 plasmid measurements for control sample stored at 4°C.

Measurement of heat-stressed pDNA samples

pDNA samples were stored at 40°C and 60°C for 1 week and measured using the LabChip Plasmid DNA Assay. Example overlay electropherograms of the stressed-sample response are shown in Figure 2. Treatment of the samples at 40°C for 1 week showed a significant increase in the amount of open-circular conformation. The average percentage purity of the supercoiled fragment decreased from 88.11% in the control to 64.60 in the 40°C group. For the degraded SC sample at 40°C, the inter-run CV of the SC main peak was 1.9%. Treatment of the samples at 60°C for 1 week showed further degradation as supercoiled conformation was completely degraded and only the linear and open-circular conformations were observed. The open-circular conformation of the 60°C stored samples was 66.30 ± 6.54%, corresponding to an inter-run CV of ~10%. Table 1 provides the percentage purity measured for each group of samples stored at 4°C and 40°C.

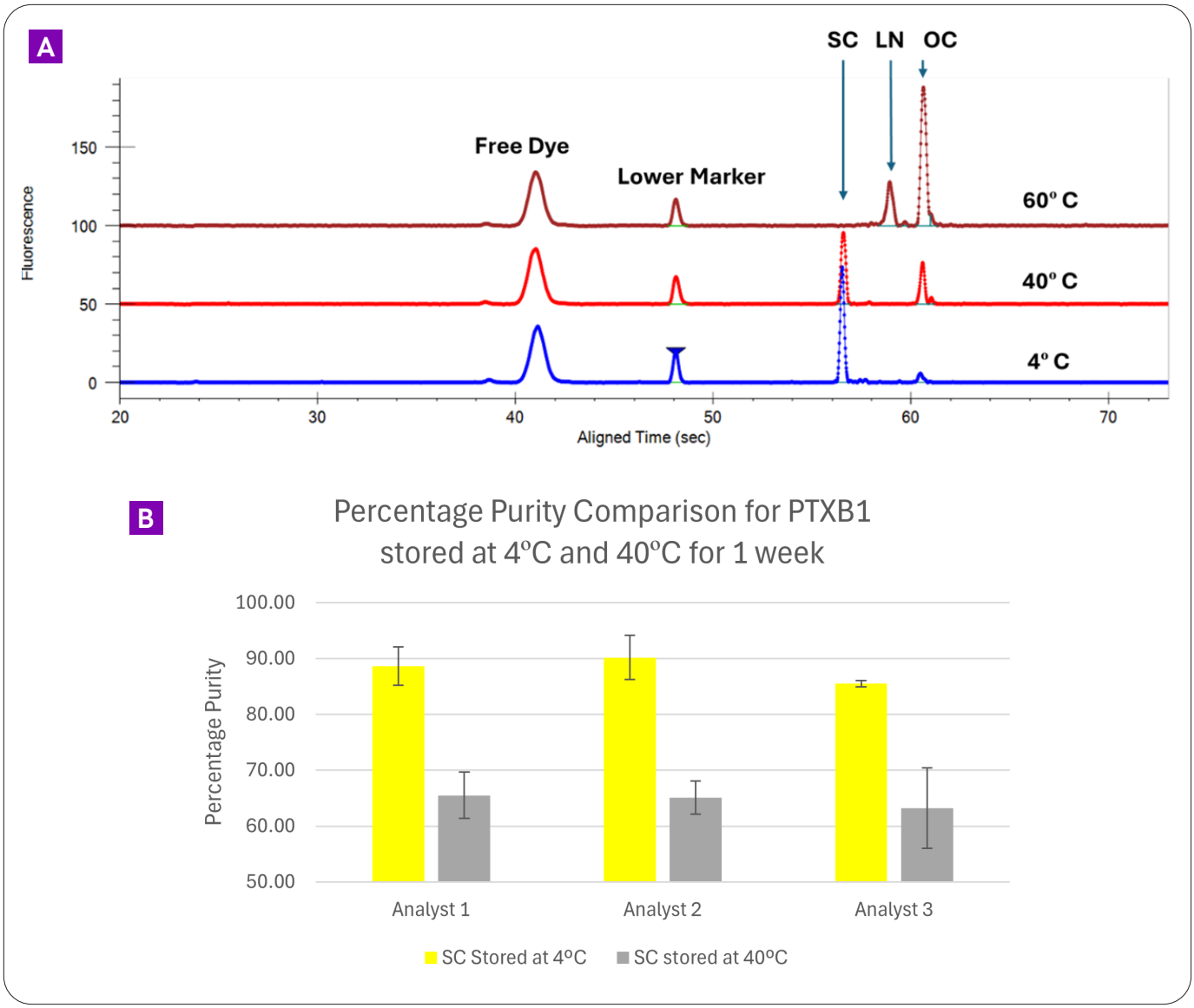

Figure 2: A) Overlay electropherograms of PTXB1 for control (4°C), heat-treated (40°C) and heat-treated (60°C) samples. The 40°C heat-treatment generated open-circular isoforms. Higher temperature heating at 60°C yielded open-circular and linear pDNA isoforms. B) Percentage purity measurements for the supercoiled pDNA main peak for 4°C and 40°C stored samples showing the measured degradation.

Table 1: Percent purity for 4°C and 40°C stored PTXB1 plasmid DNA

Group	% Purity SC stored 4°C	St Dev. SC stored 4°C	% Purity SC stored at 40°C	St Dev. SC stored at 40°C
Analyst 1	88.67	3.43	65.51	4.12
Analyst 2	90.18	3.92	65.06	2.95
Analyst 3	85.50	0.54	63.24	7.20
Avg	88.11		64.60	
St Dev	2.39		1.20	
CV	2.71		1.86	

Measurement of freeze-thawed pDNA samples

We next compared the pDNA control samples to samples that underwent a series of freeze-thaw cycles. Samples were cycled up to 12 times using a -80°C freezer, with freeze and thaw times of 1 hour each. An example electropherogram overlay and the results of the percentage purity comparison are shown in Figure 3. In contrast to heat-stressed samples which showed significant degradation, the 6 kb pDNA sample was stable to freeze-thaw cycling. No statistically significant decrease in the percentage purity of the supercoiled fraction was observed. Table 2 shows the average percentage purity measurements for the control and freeze-thawed sample groups. Interestingly, there is a report for long-term stability showing a slow degradation rate of a pDNA sample (4). In the study, the plasmid DNA maintained the supercoiled percentage purity and plasmid function during a period of 7 years when stored at or less than -20°C. Under accelerated stressing conditions though, we found that heat treatment of the pTXB1 produced open-circular impurities first and then produced both the linear and open-circular conformations with higher temperature stress. The degradation observed with 40°C treatment is similar to that published with orthogonal methods. Taken together, this could indicate that accelerated aging studies of pDNA may overestimate the plasmid DNA degradation, however it should be noted that such degradation could be sample dependent. The LabChip Plasmid DNA Assay run on the LabChip instrument shown here provides a method to answer these questions and monitor pDNA quality.

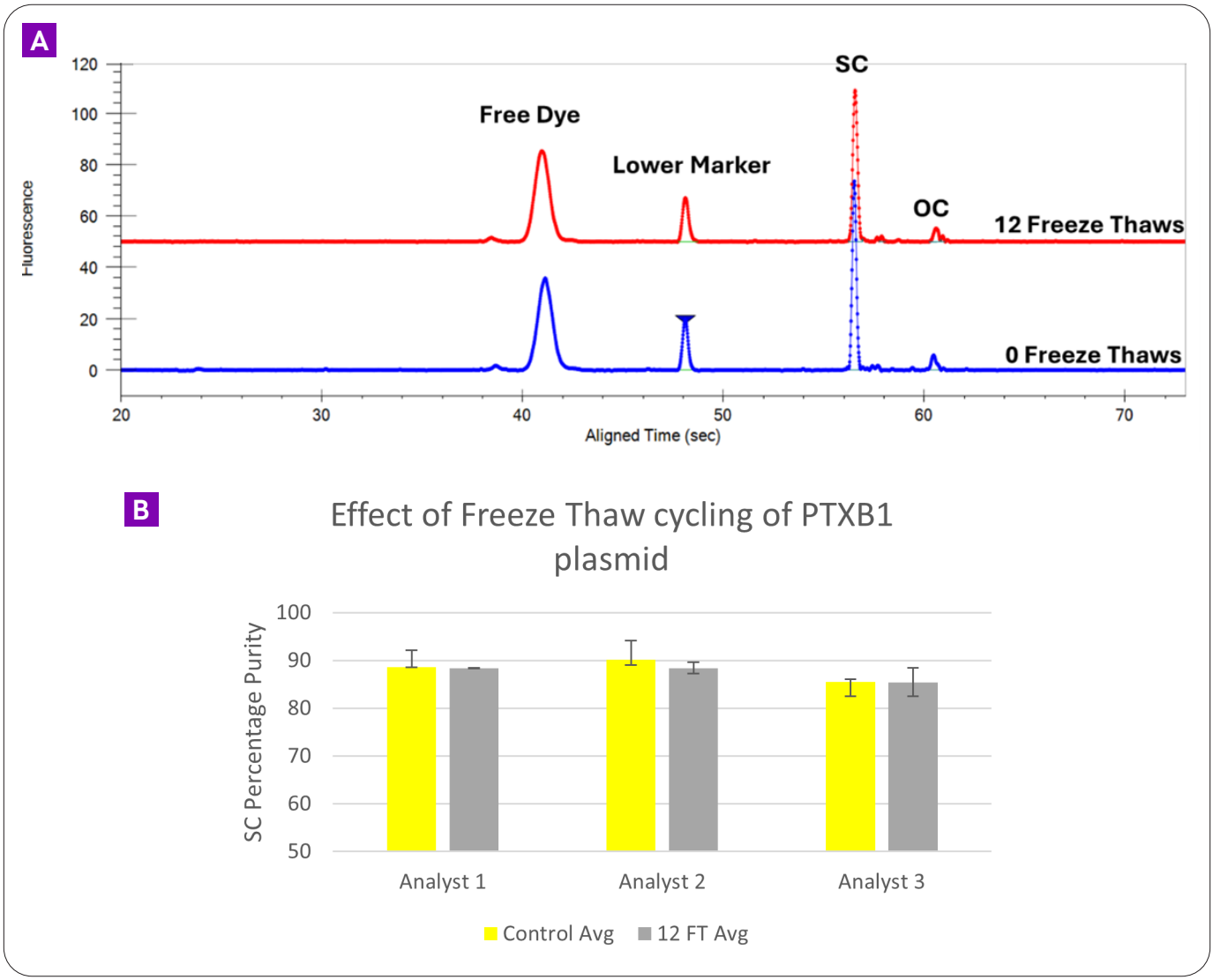

Figure 3: A) Example overlay for PTXB1 plasmid DNA with 0 and 12 freeze thaw cycles. The peak profile consisting of main supercoiled peak and small amount of open-circular impurity are very similar. B) Percentage purity of SC main peak for 0 and 12 freeze-thawed samples. The freeze-thaw group was not statistically different from the control group.

Table 2: SC Percentage purity values for PTXB1 with 0 and 12 freeze-thaw cycles

Group	% Purity 0 FT	St Dev. 0 FT	% Purity 12 FT	St Dev. 12 FT
Analyst 1	88.67	3.43	88.38	0.06
Analyst 2	90.18	3.92	88.41	1.18
Analyst 3	85.50	0.54	85.47	2.95
Avg	88.12		87.42	
St. Dev.	2.39		1.69	
CV	2.71		1.93	

Conclusions

Quality testing to monitor pDNA isoform conformation percent purity values is important to ensure pDNA efficacy. The LabChip GXII Touch system and LabChip Plasmid DNA Assay rapidly separates and measures pDNA isoforms. In this study, three first-time assay users were able to perform testing in one afternoon with SC inter-run CV less than 5%, which is within the assay specification of 10%. With the rapid sample analysis of the LabChip Plasmid DNA Assay, we were able to show that stressing samples under higher temperatures caused degradation of the intact supercoiled structures. However, freeze-thaw cycling did not have a similar effect on the plasmids. For the important task of monitoring the quality and integrity of the pDNA regularly, the LabChip Plasmid DNA Assay can be used across multiple sites for upstream and downstream analysis of pDNA. The method can be used to track stability and the quantitative analysis provides accurate and traceable results.

References

1. Lopes, A., Vandermeulen, G. & Préat, V. Cancer DNA vaccines: current preclinical and clinical developments and future perspectives. J Exp Clin Cancer Res 38, 146 (2019). https://doi.org/10.1186/s13046-019-1154-7
2. Kim, H., Danishmalik, S., Hwang, H. et al. Gene therapy using plasmid DNA-encoded anti-HER2 antibody for cancers that overexpress HER2. Cancer Gene Ther 23, 341-347 (2016). https://doi.org/10.1038/cgt.2016.37
3. Piao, X., et al. Supercoiled DNA percentage: A key in-process control of linear DNA template for mRNA drug substance manufacturing. Mol. Ther. Nucleic Acids 35, 102223 (2024).
4. Walther, W., Schmeer, M., Kobelt, D., Raier, R., Harder, A., Walhorn, V., Anselmetti, D., Aumann, J., Fichtner, I., Schleef, M., A seven-year storage report of good manufacturing practice-grade naked plasmid DNA stability, topology, and in vitro/in vivo functional analysis. Human Gene Therapy Clinical Development 24, 147-153 (2013).