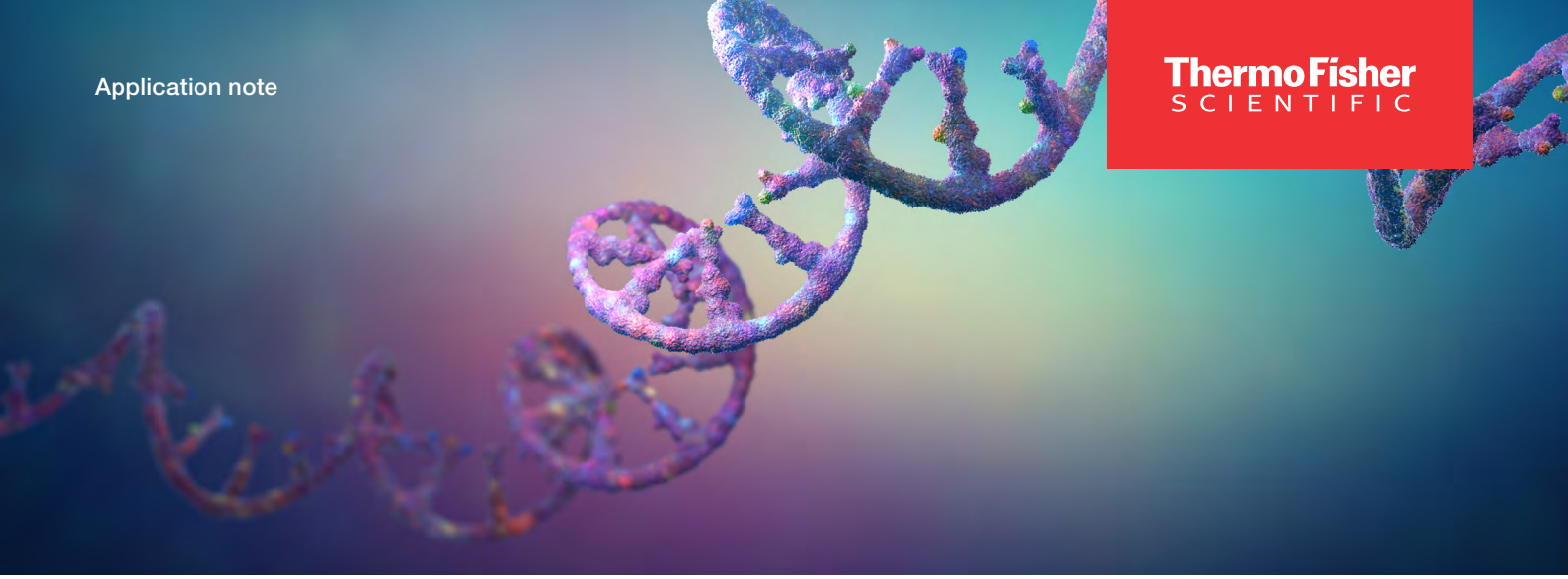
Application note



Next generation sequencing sample preparation with the NanoDrop One Spectrophotometer

Introduction

Next-generation sequencing (NGS) technology provides a genomic analysis to associate diseases such as cancer, heart disease, and diabetes with nucleic acid sequence aberrations.[1–2] NGS can detect translocations, copy number variants (CNVs), insertions and deletions (indels), and single-nucleotide variants (SNVs) with high sensitivity, all of which play a role in disease development.[3] Saliva is a common sample type for genomics research as it provides a painless, inexpensive collection procedure. Although saliva contains a plethora of proteins and microbes, human gDNA accounts for about 90% of the DNA in the sample, making it a useful sample type for downstream NGS.[4–5]

The generic NGS workflow is outlined in Figure 1. Sample preparation for NGS begins with nucleic acid extraction and purification. The purified sample then undergoes a quality and quantity assessment using spectrophotometry, fluorescence, and/or quantitative PCR (qPCR). The nucleic acid assessment ensures the concentration-dependent fragmentation parameters remain optimized for the library preparation in Step 3 (Figure 1). The sequencing library is constructed via controlled fragmentation and the newly formed fragments are then ligated to barcoding adapters that are designed specific to the platform.[1,6] The fragments are analyzed for integrity and size using various electrophoresis methods as a library preparation quality control checkpoint. The sequence is then read at each individual base as DNA synthesis occurs and application-specific data analysis is performed.[1]

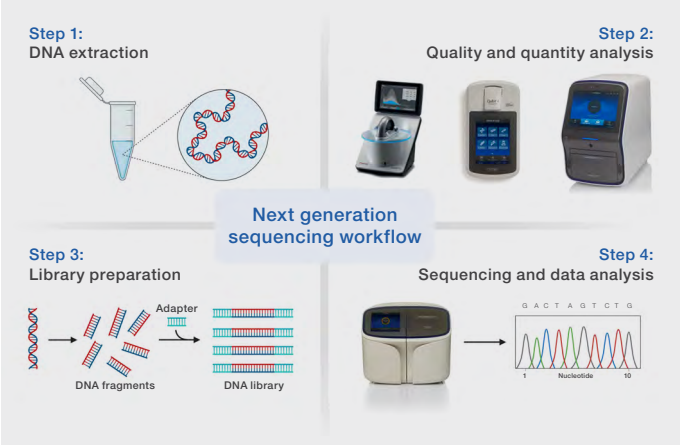


Figure 1. Workflow for NGS. Created with BioRender.com.

At present, qPCR is the most accurate quantification method available, but it is time-consuming and often requires vetting a standard operating procedure. As an alternative to qPCR, there is little agreement on whether spectrophotometry or fluorescence serves as the best method.[7–9] This is in part due to reported drawbacks of spectrophotometry: lack of absorbance specificity at 260 nm and poor sensitivity at low concentrations.[7–8] The majority of spectrophotometers available are not able to identify contaminants that co-absorb at 260 nm along with nucleic acids. The Thermo Scientific™ NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer includes the Thermo Scientific Acclaro™ Sample Intelligence Technology, which identifies common contaminants and provides a corrected nucleic acid concentration (Figure 2). The purpose of this note is to highlight the accuracy of the NanoDrop One/One^{C} Spectrophotometer in quantifying and qualifying DNA isolated from saliva for downstream NGS.

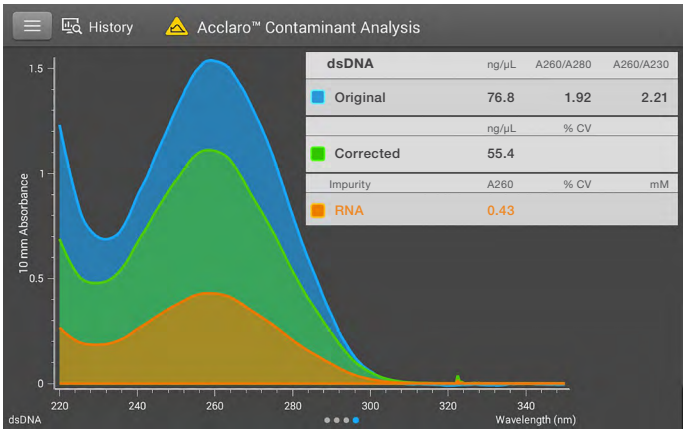


Figure 2. The Acclaro technology identifies RNA contamination (orange spectrum) in a dsDNA sample and provides a corrected dsDNA concentration (green spectrum).

Experimental procedures

DNA extraction from saliva

Human saliva was collected in a 15 mL centrifuge tube with a target of 2.5 mL, ensuring no food or drink was consumed 30 minutes prior to collection.[4] The Invitrogen™ JetFlex™ Genomic DNA Purification Kit (Invitrogen, A30700) was used to extract DNA from saliva. The extraction procedure was performed on three samples per the manufacturer's instructions with some variation to improve purity and yield. In a 1.5 mL microcentrifuge tube, 200 µL of saliva was mixed with 1.0 mL Cell Lysis Buffer and 80 µL Proteinase K; the sample was incubated at 58 °C for 1 hour. After the lysis incubation, 40 µL RNase A was added to the sample and incubated at 37 °C for 5 minutes. The sample was cooled to room temperature and 600 µL lysate was mixed with 300 µL Protein Precipitation Buffer and 100 µL Pellet Compactor; the sample was vortexed and centrifuged at 12,000 x g for 3 minutes. The supernatant was transferred to a new 1.5 mL microcentrifuge tube, an equal volume of isopropanol was added, the tube was inverted to mix, and was centrifuged at 12,000 x g for 15 minutes. Precipitating the DNA with isopropanol yields a clear DNA pellet so care was taken when the supernatant was removed after centrifugation to ensure the pellet was not inadvertently disturbed. The DNA pellet was washed twice with 70% ethanol, centrifuging after each wash for 5 minutes at 12,000 x g. The pellet was dried for 20 minutes at room temperature then resuspended in 30 µL Tris-EDTA buffer, pH 7.6. A fourth sample was extracted using the same procedure without the addition of RNase to trigger the Acclaro technology's DNA/RNA deconvolution on the NanoDrop instrument.

dsDNA assessment with the NanoDrop Spectrophotometer

The purified stock DNA samples were measured in 2 µL aliquots using the pedestal on a NanoDrop One instrument. The dsDNA application calculates concentration via the Beer-Lambert Law, using the measured absorbance at 260 nm and an extinction coefficient of 0.020 $(\mu g/mL)^{-1}$ cm^{-1}. The Acclaro technology was automatically applied to each measurement to identify common extraction contaminants and ensure a pure dsDNA sample. The mammalian DNA/RNA deconvolution was manually turned on in the dsDNA setup prior to measurements to identify RNA contamination. Stock samples were measured in replicates of five and the concentration was averaged. From the calculated average concentration, the samples were diluted to ~10 ng/µL and measured in replicates of five on a NanoDrop One instrument. For the sample that was not treated with RNase, the Acclaro correction was applied, which provided an original and corrected concentration of dsDNA; five replicates were measured. The sample was diluted to ~1 ng/µL using the average, original concentration and the average, corrected concentration to prepare for qPCR.

Quantitation via the qPCR RNase P assay

The Applied Biosystems™ TaqMan™ RNase P Detection Reagents kit (Applied Biosystems, 4316831) was used as the quantification reference targeting the single-copy human RNase P gene. The reaction was performed by an Applied Biosystems QuantStudio™ 6 Pro Real-Time PCR System and the TaqMan Fast Advanced Master Mix (Applied Biosystems, 4444557). The fast cycling protocol was performed as follows: UNG incubation at 50 °C for 2 minutes, enzyme activation at 95 °C for 2 minutes; for 40 cycles, denaturation at 95 °C for 1 second, and annealing/extension at 60 °C for 20 seconds. The RNase P kit included a human gDNA standard (10 ng/µL) that served as the template for the standard curve; the standard was serially diluted 1:1 to create 7 standards ranging from 5 ng/µL to 0.078 ng/µL. The dsDNA samples extracted from saliva were diluted from ~10 ng/µL to 1.0 ng/µL using the average concentration from the NanoDrop One instrument. Each standard and sample were assayed in triplicate and the qPCR quantification cycle (C_q) data analysis was performed using the Thermo Fisher Connect™ Design and Analysis application. The final C_q data was averaged from two qPCR runs.

Results

The sensitivity of the NanoDrop One instrument in measuring low concentrations of dsDNA is demonstrated in Figure 3. The concentrations of all three samples provided by the NanoDrop instrument were not significantly different from the TaqMan RNase P results (two-sided *t*-test assuming unequal variances; P > 0.05). The average concentrations provided by

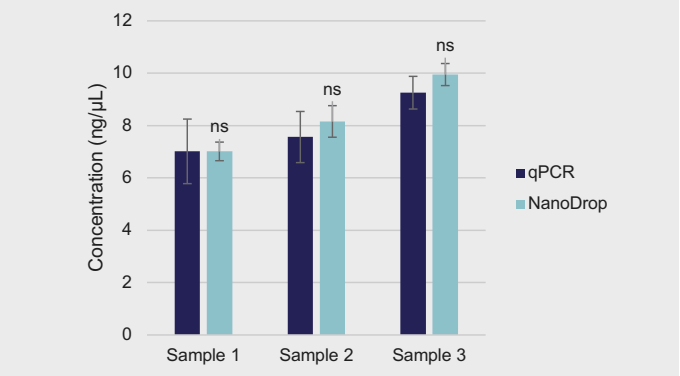


Figure 3. Comparative analysis of dsDNA concentration derived from the NanoDrop instrument (n = 5) and the TaqMan RNase P assay (n = 6) for three saliva samples. Error presented as mean ± SD; "ns" indicates not significant, two-sided P value > 0.05 with the unequal variances *t*-test.

the NanoDrop instrument for samples 1–3 were 7.01 ± 0.36, 8.16 ± 0.60, and 9.95 ± 0.421 ng/µL, respectively. The RNase P assay served as the reference; the average concentrations for samples 1–3 were 7.01 ± 1.23, 7.56 ± 0.977, and 9.26 ± 0.624 ng/µL, respectively, based on two qPCR runs.

One sample was not treated with RNase to trigger the Acclaro DNA/RNA deconvolution feature of the NanoDrop One/One^{C} Spectrophotometer. The original, uncorrected concentration from the NanoDrop instrument was 78.24 ± 0.91 ng/µL and the corrected concentration was 58.33 ± 2.10 ng/µL, averaged from 5 replicates. The sample was diluted for the TaqMan RNase P assay using the original and corrected concentrations separately to determine the effect on the C_q. Typically, a higher C_q equates to a lower amount of template, or a lower concentration, and the opposite is true for a lower C_q. Outlined in Table 1, when the dilution calculations included the corrected concentration, the C_q value was 29.28. When the dilution calculations included the original, uncorrected concentration from the NanoDrop instrument, the C_q value increased to 31.50, indicating a lower amount of template since the sample was over-diluted. The resulting change in C_q (ΔC_q) is 2.22 and, since qPCR cycles are logarithmic, the fold change equates to 0.21 ($2^{-2.22}$) assuming 100% assay efficiency.[10] Without the DNA/RNA deconvolution feature on the NanoDrop One instrument, the scientist would incorrectly dilute the sample because RNA and DNA co-absorb at 260 nm. For successful NGS, the key takeaway is to appropriately clean up the dsDNA sample, which is made easier by identifying the contaminant with the Acclaro technology and the DNA/RNA deconvolution feature.

	Original concentration	Corrected concentration
NanoDrop (ng/µL)	78.24	58.33
C_q	31.50	29.28

Table 1: Changes in C_q (n = 3) from diluting based on the original and corrected concentrations from the NanoDrop instrument (n = 5).

Conclusions

Currently, quantifying dsDNA prior to preparing an NGS library is not standardized, causing inconsistencies between studies.[7] In this note, the NanoDrop One/One^{C} Spectrophotometer has shown to be a reliable and sensitive method for quantifying low concentrations of dsDNA upstream of NGS. When compared to the concentration determined by qPCR, the NanoDrop One instrument reported a concentration that was not significantly different. The Acclaro technology provides a nucleic acid quality assessment by identifying common extraction contaminants and deconvolutes RNA from a DNA spectrum, making sample clean-up and protocol optimization simpler. As an accurate concentration and a high-quality DNA sample are required for a successful NGS run, the NanoDrop One/One^{C} Spectrophotometer is a full-service, dependable instrument that is easily implemented into the NGS workflow.

References

1. Thermo Fisher Scientific (n.d.). What is Next-Generation Sequencing? https://www.thermofisher.com/us/en/home/life-science/sequencing/sequencing-learning-center/next-generation-sequencing-information/ngs-basics/what-is-next-generation-sequencing.html
2. National Human Genome Research Institute (2020). DNA sequencing fact sheet. https://www.genome.gov/about-genomics/fact-sheets/DNA-Sequencing-Fact-Sheet
3. Thermo Fisher Scientific (2023). Introduction to next-generation sequencing: Ion Torrent technology and instrumentation. http://assets.thermofisher.com/TFS-Assets/CSD/Reference-Materials/next-generation-sequencing-ebook.pdf
4. Goode, M. R., Cheong, S. Y., Li, N., Ray, W. C., & Bartlett, C. W. (2014). Collection and Extraction of Saliva DNA for Next Generation Sequencing. Journal of Visualized Experiments : JoVE, 90, 51697. https://doi.org/10.3791/51697
5. Marotz, C. A., Sanders, J. G., Zuniga, C., Zaramela, L. S., Knight, R., & Zengler, K. (2018). Improving saliva shotgun metagenomics by chemical host DNA depletion. Microbiome, 6, 42. https://doi.org/10.1186/s40168-018-0426-3
6. Mohideen, A. M. S. H., Johansen, S. D., & Babiak, I. (2020). High-Throughput Identification of Adapters in Single-Read Sequencing Data. Biomolecules, 10(6), 878. https://doi.org/10.3390/biom10060878
7. Dang, J., Mendez, P., Lee, S., Kim, J. W., Yoon, J.-H., Kim, T. W., Sailey, C. J., Jablons, D. M., & Kim, I.-J. (2016). Development of a robust DNA quality and quantity assessment qPCR assay for targeted next-generation sequencing library preparation. *International Journal of Oncology*, *49*(4), 1755–1765. https://doi.org/10.3892/ijo.2016.3654
8. Bruijns, B., Hoekema, T., Oomens, L., Tiggelaar, R., & Gardeniers, H. (2022). Performance of Spectrophotometric and Fluorometric DNA Quantification Methods. Analytica, 3(3), Article 3. https://doi.org/10.3390/analytica3030025
9. Robin, J. D., Ludlow, A. T., LaRanger, R., Wright, W. E., & Shay, J. W. (2016). Comparison of DNA Quantification Methods for Next Generation Sequencing. Scientific Reports, 6, 24067. https://doi.org/10.1038/srep24067
10. Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods, 25(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Learn more at **thermofisher.com/nanodrop**

thermo scientific