

Microvolume Quantification of Nucleic Acids in Molecular Diagnostics

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Abstract

Quantification of nucleic acid samples prior to analysis is an important QC step in order to verify suitability for downstream testing. Assessing quantity and purity of samples prior to analysis has great value because of the financial and time costs of analytical failures. Clinical diagnostic laboratories frequently work with limited amounts of biological samples; therefore not only is the resultant nucleic acid yield often small, but the opportunity to re-isolate nucleic acids is limited.

The Thermo Scientific NanoDrop 2000c and NanoDrop 8000 present several key advantages over conventional spectrophotometers, particularly the capability to quantify nucleic acids in as little as a single microliter. Analysis takes less than five seconds per sample, while the exceptional dynamic range of the instruments means that sample dilution is not necessary. Here we describe the necessity of microvolume quantification of nucleic acids prior to downstream molecular diagnostic applications such as qPCR, RT-qPCR, STR analysis, SNP genotyping and DNA sequencing, and also describe how the NanoDrop™ technology provides unique advantages over other quantification methods.

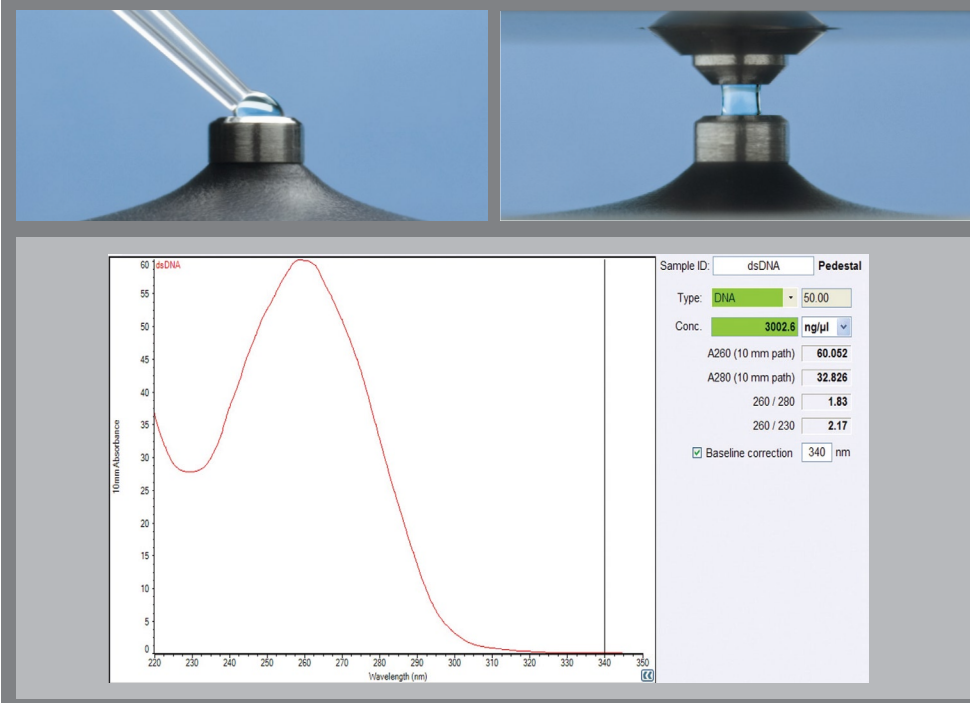
NanoDrop UV-Vis Spectrophotometers

NanoDrop UV-Vis spectrophotometers utilize a revolutionary sample retention technology which retains 1 µL samples in place via surface tension between two fiber optic cables (fig. 1). After measurement, samples are quickly and easily removed from the optical surfaces with an ordinary dry laboratory wipe.

The NanoDrop 2000/2000c spectrophotometer utilizes multiple pathlengths (1.0 mm, 0.2 mm, 0.1 mm and 0.05 mm) that change in real time while measuring a 1 µL sample, resulting in a wide dynamic range capable of measuring 2 - 15,000 ng/µL of dsDNA. In contrast to this, measuring samples with a standard 10 mm quartz cuvette on a conventional spectrophotometer typically has an upper detection limit of 50 ng/µL with a minimum sample volume of 1 mL. Moreover, the use of cuvettes can potentially lead to cross-contamination from prior samples if not properly cleaned.

NanoDrop users also benefit from a quick measurement cycle. The total cycle time for the NanoDrop 2000c is less than five seconds, and in cases where higher throughput is required, the NanoDrop 8000 can measure up to eight samples at a time with a total cycle time of just 20 seconds. The intuitive software displays the calculated DNA concentration, nucleic acid purity ratios, and spectra of each sample (fig. 1).

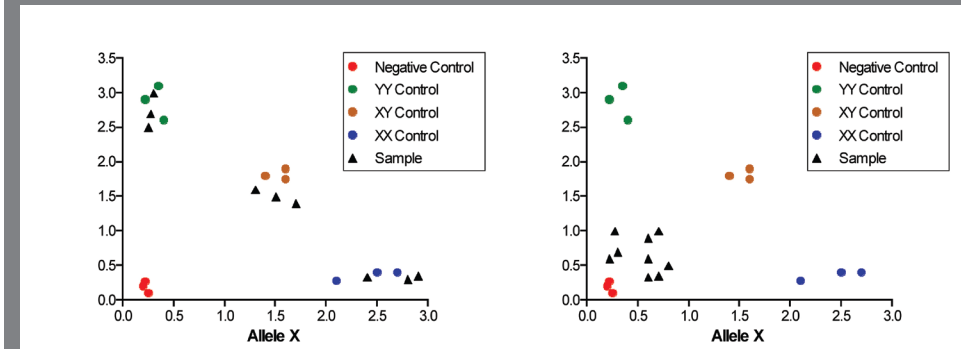
Figure 1 Left: loading of a 1 µL sample on the measurement pedestal. Right: sample measurement. Lower panel: software output, showing both spectra and numerical data.



Quantification

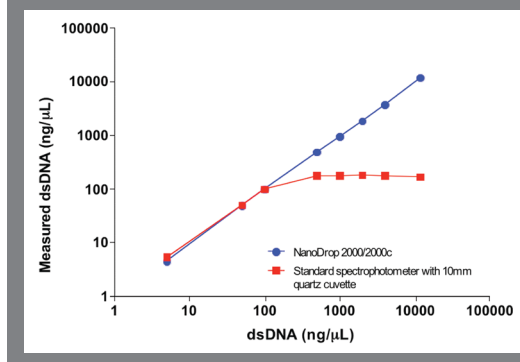
Molecular techniques used in clinical diagnostics today are often qPCR based assays. When conducting assays involving absolute quantification, it is important that unknown samples lie within the standard curve. Extrapolating standard curves for outliers is a hazardous practice, as PCR efficiency may be reduced with excess template loading, while background or contamination may be mistaken for low template quantities. Even genotyping and sequencing reactions, although not quantitative by nature, may fail to give necessary signal if template quantities are not optimized (fig. 2).

Figure 2 Effect of low template quantity on SNP genotyping. Appropriate template quantity (left) results in easy identification of genotypes, while low template quantities (right) make allele calls unreliable or impossible.



Typical assay input DNA concentrations for qPCR, SNP genotyping, STR analysis or sequencing reactions range from 2 – 200 ng/µL dsDNA; however extracted sample concentrations may be outside of this range. The pathlength optimization and microvolume capability of the NanoDrop sample retention system increases the measurable concentration range 300 fold compared to a standard spectrophotometer using a 10 mm pathlength cuvette (fig. 3). This automatic pathlength adjustment facilitates the direct quantification of concentrated samples without the need for sample dilutions.

Figure 3 Dynamic range when measuring dsDNA using both a NanoDrop 2000c and a spectrophotometer utilizing a classic 10 mm quartz cuvette.



Purity

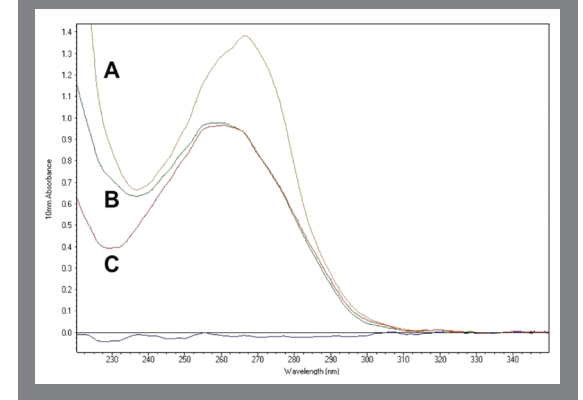
Most common nucleic acid extraction techniques begin by lysing cells, followed by separation of nucleic acids from other cellular macromolecules. Residual quantities of chemicals used in either the initial cell lysis or purification steps can occasionally be found in the final purified sample, where they have the potential to negatively influence downstream applications. Many of these contaminants can be detected using UV/Visible spectrophotometry, therefore aberrant spectra may be evidence of contamination. Examination of the spectral data for each nucleic acid sample often aids in troubleshooting and may sometimes aid in the identification of contaminants before downstream analysis.

Phenol is an organic solvent commonly used for the removal of protein by organic-aqueous phase separation. Phenol contamination in extracted nucleic acid samples typically arises during the removal by pipette of the aqueous phase from the organic phase. Phenol contamination is often conspicuous as it shifts the 230 nm minima and 260 nm maxima of the dsDNA spectra to 240 nm and 270 nm, respectively (fig. 4). It is also noteworthy that phenol results in a significant contribution at 260 nm, causing overestimation of DNA concentration (fig. 4).

Similarly, chaotropic salts such as guanidine used in some spin column methods can also become a contaminant if there is too little DNA bound to the silica membrane. In these cases, salts are not removed during wash steps, but rather

are eluted with nucleic acids at the end of the extractions. Typically, chaotropic salts lead to significant absorbance at 230 nm (fig. 4), resulting in an apparent shift of the minima found at this wavelength to a higher wavelength. Depending on concentration, it is also possible that chaotropic salts will influence apparent DNA concentration. The signal from residual contaminants can contribute to overestimation of DNA concentration and in the worst cases completely mask the nucleic acid signal. Examination of spectra, however, can in some cases alert users to potential contaminants that may negatively impact downstream applications. Additionally, spectral distortion can also be described by the well established 260/230 and 260/280 purity ratios reported by the software.

Figure 4 Spectra of DNA (A) contaminated with phenol, (B) contaminated with guanidine, and (C) without contamination.



Sample Quality Control in Molecular Diagnostics

Techniques such as qPCR, SNP genotyping, STR analysis and DNA sequencing are powerful genotyping tools; however they are prone to failure when amounts and purity of template are not optimal. Although these methods are increasingly robust, nucleic acid extractions often contain unexpected yields, as well as various contaminants that may interfere with downstream analysis.

The time and financial costs of failed sample analyses can be significant. QC of template quantity means that delays caused by failed nucleic acid extractions are limited to the time required to re-extract samples, which is typically less than an hour. However, if failed DNA extractions are not identified, the delay while accumulating sufficient samples for analysis may delay the reporting of results by as much as 1 – 3 weeks in medium to small sized facilities.

Applying this paradigm of nucleic acid quality control post-DNA extraction to molecular diagnostics workflows reduces analysis failures, resulting in financial savings and reduced delays in patient diagnoses. NanoDrop spectrophotometers represent a fast and simple QC method for assessing nucleic acid samples.

The primary driver for quality control measurements post-DNA extraction is to increase confidence in downstream analyses. This confidence is attributable to the following factors, especially when using a microvolume method for measuring sample concentration and purity:

- Fast measurement: Direct measurement of concentrated samples eliminates sample dilution time
- Reduction in errors: Direct sample measurement removes errors introduced when diluting samples
- Purity assessment: Monitoring of sample purity by 260/230 and 260/280 ratios and spectral data
- Fewer analysis failures: Reduction in financial costs of failed reactions and associated delays in reporting patient results
- Ease of use: Straightforward measurement process and display of the results within five seconds

Conclusions

The financial cost of failed downstream analysis can be significant; however even more significant is the additional delay in reporting results to physicians and patients, which may be as much as 1-3 weeks. Quantification of DNA and assessment of potential contaminants post-extraction are therefore highly worthwhile quality control procedures. Adding this small step to the established workflow will result in increased confidence in the downstream experimentation. The patented microvolume sample retention system used in NanoDrop spectrophotometers prevents cross-contamination, allows a far greater concentration range to be measured and reduces the length of time needed for analysis. Samples can be analyzed in seconds without the need for dilutions or cuvettes, making this a fast and simple addition to the sample analysis workflow.

For more information: www.thermo.com/nanodrop