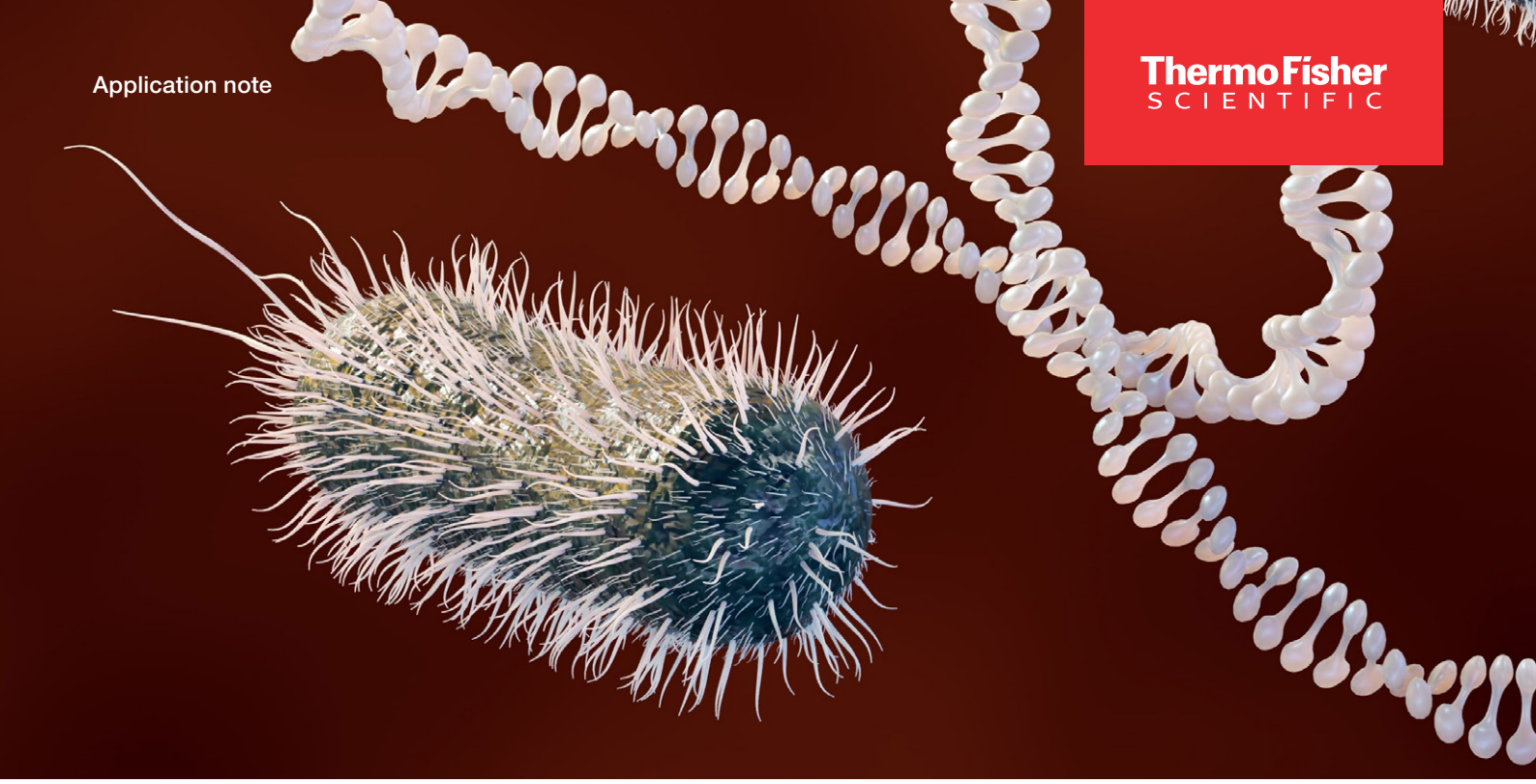

Application note

Analyze supercoiled plasmids with the NanoDrop One/One^{C} Spectrophotometer

Introduction

Plasmids are circular pieces of double-stranded DNA (dsDNA) in bacterial cells that replicate independently of the bacterial genome and are used to deliver gene therapies to human cells. When manufacturing plasmids, purity and concentration quality control measurements are performed to maintain consistency between batches and to maintain safety and efficacy.[1] To perform such measurements, ultraviolet-visible (UV-Vis) spectrophotometry is a fast and simple technique that can be easily implemented at several points in the plasmid manufacturing workflow.

Plasmid Manufacturing

The three main steps of plasmid manufacturing are outlined in Figure 1 with the incorporation of the Thermo Scientific™ NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer. In Step A, vector preparation, the plasmid cloning vector designed for use in gene therapies requires several elements: an origin of replication; the *lacZ* gene; one or multiple gene(s) of interest; and an antibiotic resistance gene for selection.[2] The vector is digested with restriction enzymes to generate compatible ends with the target gene insert, and gel electrophoresis is used to analyze and purify the fragments of interest.[3] After purifying the fragments from the gel, the NanoDrop One Spectrophotometer is used to verify purity and assess concentration of the fragments to ensure a successful ligation reaction.

The transformation in Step B includes a heat shock at 42°C of chemically competent *E. coli* to increase uptake of the ligated plasmid DNA.[4] The transformation mix is then spread on an antibiotic agar plate containing the chromogenic substrate X-gal to screen for blue and white colonies. If the ligation is successful, the colonies appear white due to the disruption of the *lacZ* gene.[5] In Step C, the white colonies are used to inoculate antibiotic-containing growth media, the culture is incubated overnight, and the optical density at 600 nm (OD_{600}) is measured on the NanoDrop One Spectrophotometer.[1,6] The plasmid DNA is extracted from the *E. coli* cells and the NanoDrop One instrument is used to perform a final purity and concentration check prior to downstream applications.[1]

Nucleic Acid Purity

A plasmid that will be used for therapeutics requires the purified plasmid to be free of contaminating proteins as these may cause adverse side effects to the patient.[7] Traditionally, the A260/A280 purity ratio has been used to gauge protein contamination in nucleic acids, where 1.8 is typical for “pure” DNA and 0.6 for “pure” protein. However, due to the small extinction coefficient of most proteins, a high percentage of protein (~50%) is required to significantly influence the nucleic acid purity ratio.[8] Built into the NanoDrop One software, the Thermo Scientific™ Acclaro™ Sample Intelligence Technology overcomes this hurdle by identifying and approximating the amount of protein contamination in nucleic acid samples, eliminating the guesswork of analyzing purity ratios.

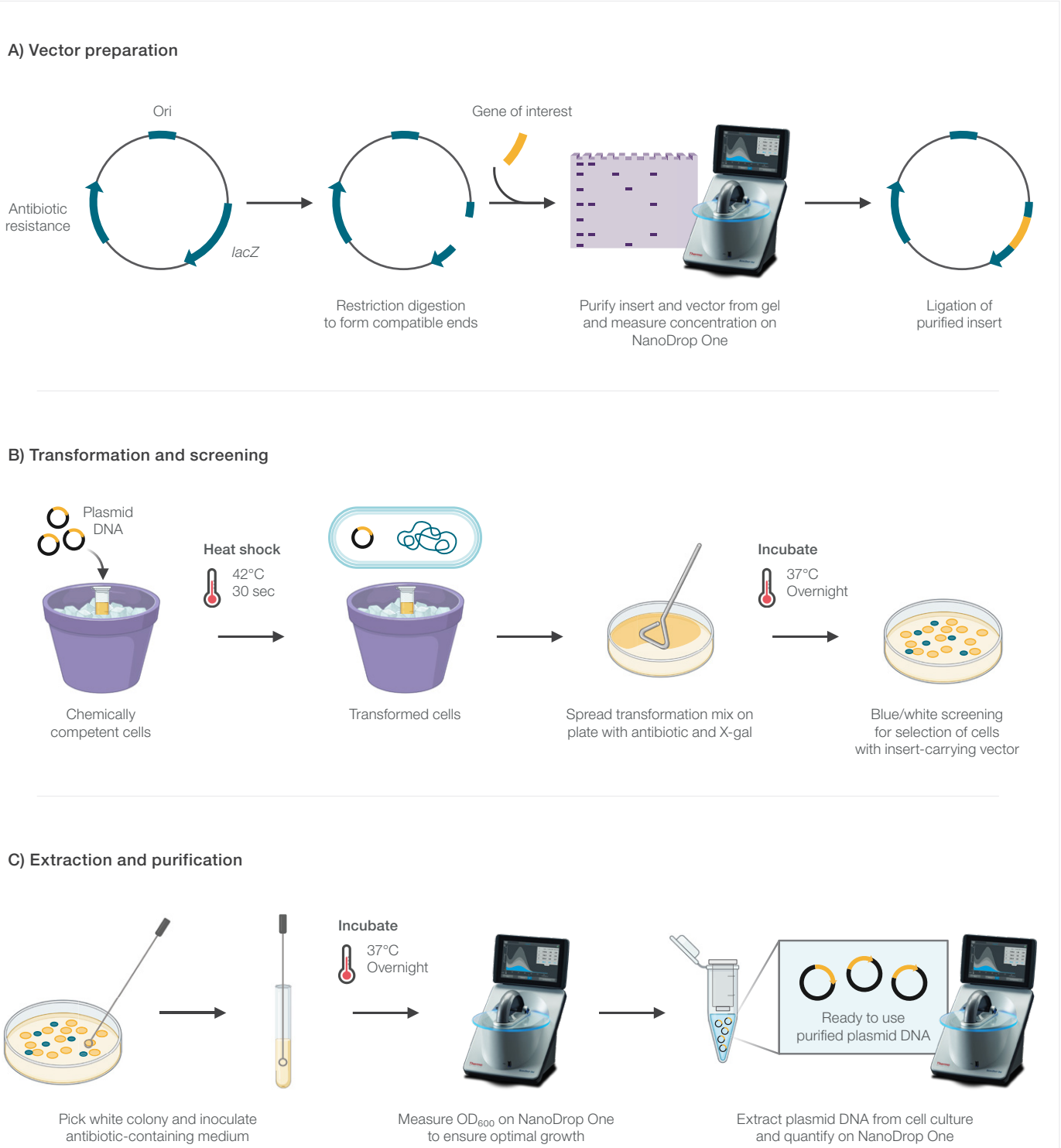

Figure 1. Steps for developing plasmid DNA and the implementation of the NanoDrop One Spectrophotometer: A) preparing a plasmid cloning vector, B) transforming *E. coli* cells and their subsequent selection, and C) extraction and quantification of plasmid DNA. Figure created with BioRender.com.

Supercoiled Plasmid DNA

The DNA topology is important to identify for efficient transfection of the gene therapy plasmid. Of the different topologies—linear, nicked, and supercoiled—a supercoiled plasmid exhibits optimal transfection efficiency.[9-10] After the plasmid is isolated and purified, gel electrophoresis is useful in defining the plasmid topology. A tightly wound, supercoiled plasmid migrates through the gel pores much faster than a nicked or linear plasmid, making the band appear farther down the gel.[11-12] A nicked plasmid, also referred to as open circular, will migrate more slowly and remain towards the top of the gel. A linear plasmid band will remain in-between a nicked and supercoiled plasmid band. It is common to visualize multiple bands for plasmids as nicking of the plasmid can easily occur during purification.[11]

Quantifying DNA with UV-Vis spectrophotometry and the Beer-Lambert Law is based on the absorbance of the aromatic rings present in the nitrogenous base of purines and pyrimidines. Supercoiled DNA is prone to a hypochromic shift due to the stacking orientation of the nitrogenous bases, seen as a decrease in the absorption spectrum (Figure 2).[13] As DNA concentration is proportional to the absorbance based on Beer's Law, the reported concentration also appears lower than expected.

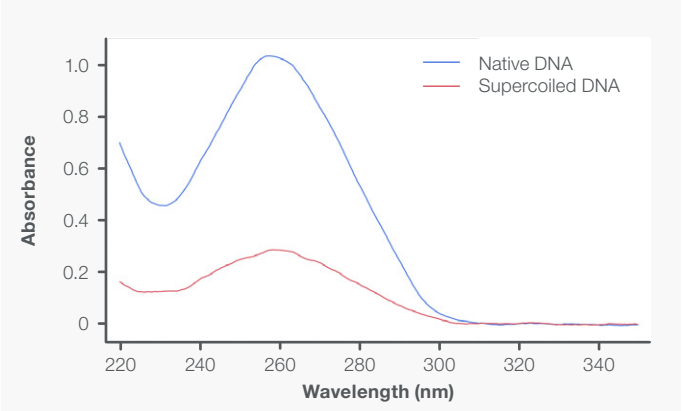

Figure 2. Hypochromic shift of supercoiled DNA (red line) compared to native DNA (blue line).

To overcome the hypochromic effects seen in supercoiled plasmid DNA, the DNA can be relaxed by performing a treatment with heat, topoisomerase, restriction endonucleases, or NaOH.[14-16] This step of relaxing DNA is also necessary for downstream sequencing or PCR to allow primers and enzymes proper access to the DNA.[15] If an enzymatic method is utilized, the DNA must be purified from the contaminating proteins to ensure an accurate absorbance measurement with UV-Vis spectrophotometry.

Experimental Procedures

Transformation

Prior to transformation, X-gal solution (Thermo Scientific, R0941) was spread onto three LB agar plates supplemented with 50 µg/mL streptomycin (Thermo Scientific, J62665EQF) and diffused for one hour. The plates were then warmed in an incubator for 30 minutes at 37°C. Plasmid DNA (pUC19) was transformed into *E. coli* cells as supplied by the Invitrogen™ One Shot™ TOP10 Chemically Competent *E. coli* kit (Invitrogen, C404010). The transformation procedure was performed as per the One Shot protocol in triplicate. The final mixtures were spread on the LB plates in 100 µL aliquots. The plates were then inverted and incubated at 37°C overnight. A single white colony was picked per plate and was used to inoculate 10 mL LB broth supplemented with 50 µg/mL streptomycin. The cultures were incubated overnight at 37°C, shaking at 300 rpm. A negative control transformation plate and culture tube were included to account for inadvertent contamination.

OD_{600} and DNA Extraction

The optical density at 600 nm (OD_{600}) was determined using the microvolume pedestal on a NanoDrop One Spectrophotometer, utilizing the OD_{600} application and a cell number conversion factor of 1 x 10^8 cells/mL. A 2.0 µL aliquot of LB broth was used to blank the spectrophotometer and cell culture samples were measured in triplicate using 2.0 µL on the pedestal surface. Plasmid DNA was extracted from each culture tube using the Thermo Scientific GeneJET™ Plasmid Midiprep Kit (Thermo Scientific, K0481) following "Protocol A: plasmid DNA purification using low speed centrifuges." DNA was eluted with 150 µL of Elution Buffer to increase the sample concentration. A 2.0 µL aliquot of Elution Buffer was used to blank the NanoDrop One Spectrophotometer and samples were measured in triplicate using 2.0 µL on the pedestal surface and the dsDNA application.

Quantifying Supercoiled Plasmid DNA

Relaxed pHOT1 DNA was supercoiled using the TopoGEN *E. coli* DNA Gyrase and Relaxed DNA Assay Kit (TopoGEN, TG2000GKIT). The assay was carried out as described in the kit protocol with several modifications. To supercoil the plasmid, 5.0 µL of relaxed pHOT1 was mixed with 4.0 µL buffer, 5.0 µL gyrase, and 6.0 µL deionized (DI) water. The mixture was incubated for 30 minutes at 37°C. To purify the supercoiled plasmid DNA from the contaminating proteins and salts and stop the gyrase reaction, a chloroform extraction was performed. The volume of the supercoiled DNA mixture was brought up to 200 µL with DI water and 200 µL of chloroform - isoamyl alcohol (24:1) was added for phase separation. The extraction mixture was vortexed and centrifuged at 11,000 rpm for 5 minutes and the top aqueous layer was withdrawn. To this layer, 2/3 volume of room temperature isopropanol and 0.08 volume of 3.0 M sodium acetate were added and mixed by inversion. The precipitation was performed for one hour at room temperature then centrifuged at 11,000 rpm for 15 minutes. The pellet was washed with 1.0 mL 70% ethanol and centrifuged at 11,000 rpm for 10 minutes. After removing the supernatant, the pellet was air dried for 20 minutes then resuspended in 30 µL tris-EDTA pH 7.6.

Gel electrophoresis was performed on the relaxed and supercoiled pHOT1 using the Lonza™ FlashGel™ System and the corresponding 1.2% agarose DNA cassettes. After confirming the presence of supercoiled DNA with the gel, the relaxed and supercoiled pHOT1 were quantified in triplicate on the NanoDrop One Spectrophotometer using the dsDNA application and the microvolume pedestal to compare the absorbance intensities. The supercoiled pHOT1 was then relaxed by heating to 90°C for 5 minutes and again quantified with the NanoDrop One instrument to reduce the hypochromic effect of supercoiled DNA.

Results

The measured OD_{600} for all three samples and the negative control are outlined in Table 1 with the corresponding standard deviation. The GeneJET plasmid midiprep kit required an optical density of 2 – 3 at 600 nm and all samples fell within that range at an average of 2.72 OD, except for the negative control. An optical density of 2 – 3 is not within the detection limits of most cuvette-based spectrophotometers but the auto-ranging pathlength function with the NanoDrop One instrument pedestal provides accurate OD_{600} measurements greater than 1.0 OD. The default cell number conversion factor in the NanoDrop One software is 1×10^8 cells/mL, which is a generally accepted factor for most bacterial cells such as *E. coli*. Using this factor in Beer's Law, the cell densities for Samples 1 – 3 are 2.70×10^8, 2.77×10^8, and 2.69×10^8 cells/mL, respectively.

Sample Name	OD_{600}	Standard Deviation (OD)
Sample 1	2.70	0.02
Sample 2	2.77	0.01
Sample 3	2.69	0.05
Negative Control	0.02	0.01

Table 1. Average OD_{600} data (n = 3) and standard deviation measured on the NanoDrop One Spectrophotometer microvolume pedestal.

The three purified plasmid DNA sample concentrations are shown in Table 2 with the corresponding standard deviations. The sample concentrations ranged from 6.73 ng/µL – 10.71 ng/µL with standard deviations below 0.19 ng/µL, indicating excellent reproducibility for measuring lower concentration samples.

Sample Name	Concentration (ng/µL)	Standard Deviation (ng/µL)
Sample 1	6.73	0.16
Sample 2	10.71	0.19
Sample 3	9.77	0.11

Table 2. Average (n = 3) concentrations and standard deviation of purified plasmid DNA measured on the NanoDrop One Spectrophotometer microvolume pedestal.

An image of the gel electrophoresis result is shown in Figure 3, which confirmed the experimental procedure with gyrase successfully produced supercoiled DNA. Supercoiled pHOT1 traveled farther on the gel than relaxed pHOT1 because the supercoiled topology allows for a faster gel migration.[11-12] A high resolution gel system will be able to further resolve the topoisomers expected to be present in the relaxed plasmid DNA sample.

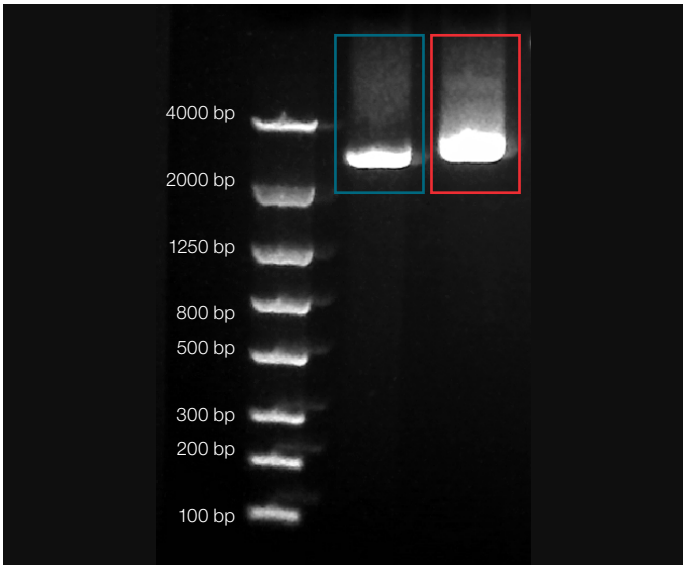

Figure 3. Gel electrophoresis of supercoiled (blue box) and relaxed (red box) pHOT1 using 1.2% agarose gel.

The supercoiled, relaxed, and heat-treated pHOT1 concentration results are provided in Table 3. The relaxed pHOT1 served as the control with an average concentration of 51.23 ng/µL. Supercoiled pHOT1 displayed a hypochromic shift, which resulted in a reduced concentration of 14.10 ng/µL. When the supercoiled pHOT1 was heat-treated, the concentration returned to 55.78 ng/µL. With heat-treatments, too high of a temperature will denature the dsDNA into single strands, causing a hyperchromic shift and an increase in concentration. It is important to perform DNA melting experiments to properly identify the melting temperature at which dsDNA denatures into ssDNA.[17]

Sample Name	Concentration (ng/µL)	Standard Deviation (ng/µL)
Relaxed pHOT1	51.23	0.47
Supercoiled pHOT1	14.10	0.33
Heat-treated pHOT1	55.78	1.51

Table 3. Average (n = 3) concentrations and standard deviation of relaxed, supercoiled, and heat-treated pHOT1 measured on the NanoDrop One Spectrophotometer microvolume pedestal.

Conclusions

UV-Vis spectrophotometry is an invaluable technique that aids in standardizing quality control checkpoints in the plasmid manufacturing workflow. The presented data illustrates the reliability of the NanoDrop One Spectrophotometer in measuring the OD_{600} of cell cultures, quantifying plasmid DNA, and comparing quantitative differences between supercoiled and native plasmid DNA. Also presented is the importance of relaxing supercoiled plasmid DNA to accurately calculate concentration with UV-Vis spectrophotometry by reducing the hypochromic effect. The key takeaway for relaxing supercoiled plasmid DNA is that a standard operating procedure must be established for the chosen relaxation method to ensure consistent and reliable quantitation results. The NanoDrop One Spectrophotometer provides full spectral data from a 1.0 - 2.0 µL sample volume, making plasmid quality control a simple step in the manufacturing process.

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