Application note

Evaluating DNA Purity for Molecular Cloning Quality Control

NanoDrop Lite Plus Spectrophotometer

Molecular cloning requires two main components to create recombinant DNA: 1) a DNA vector; and 2) one, or several, DNA fragment(s) containing the gene(s) of interest (GOI). To generate a fragment containing a GOI, digestion with a restriction endonuclease cuts the DNA at specific sites and yields base pairing complementarity with a similarly digested vector through their cohesive ends (Figure 1A). The vector and the DNA fragment are then joined at their cohesive ends by a ligase enzyme to form the recombinant DNA product (Figure 1B).[1-3]

Quality control (QC) in molecular cloning is important before and after the restriction digestion step. Once extracted from the cell line, plasmid DNA purity should be checked before beginning the digestion. Common contaminants from DNA extraction include phenol, ethanol, and salts, which are known to inhibit the restriction endonuclease and prevent proper cleavage.[4] After digestion, the efficiency can be evaluated with agarose gel electrophoresis by verifying the presence of expected bands and little to no smearing.[5]

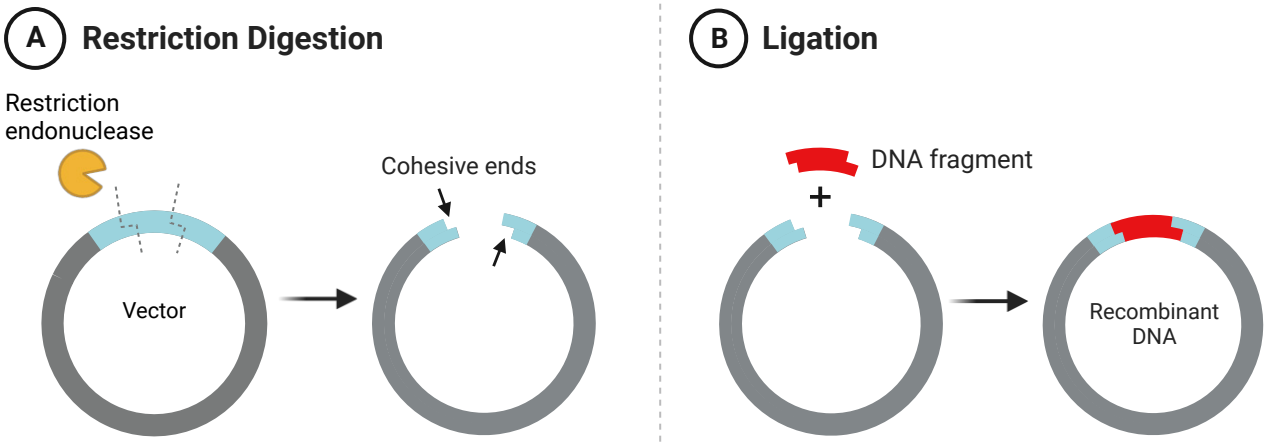

Figure 1. A) Restriction digestion of a vector with an endonuclease to produce cohesive ends for complementary binding with a DNA fragment containing a gene of interest. B) Ligation of the digested DNA fragment and vector to produce recombinant DNA. Figure created with BioRender.com.

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Using restriction endonucleases produces many fragments of varying lengths when attempting to clone a single gene from genomic DNA.[3] For this reason, restriction digested DNA is commonly analyzed via gel electrophoresis after digestion, and the correct fragment band containing the GOI can be excised and purified from the gel for downstream ligation and transformation. Purity and concentration should also be determined after gel purification to ensure highly efficient ligation and transformation.

The purity and concentration of extracted plasmid DNA is typically evaluated with ultraviolet-visible (UV-Vis) spectrophotometry as it is a quick and simple technique. DNA absorbs light at 260 nm in the UV range, while salts absorb below 230 nm and proteins and phenolics absorb around 280 nm. The measured absorbance can be related to the concentration using the Beer-Lambert Law, shown in the following equation, where "c" = concentration, "A" = absorbance at a 1.0 cm pathlength, "ε" = sample-specific extinction coefficient, and "b" = pathlength (typically 1.0 cm):

$$A = \varepsilon bc$$

The purity ratios, A260/A230 and A260/A280, are lowered by the presence of salts and proteins, respectively. This makes the ratios key tools for assessing purity of DNA. For dsDNA, the expected A260/A280 ratio is ~1.8 and the expected A260/A230 ratio range is 2.0 - 2.2.

The Thermo Scientific™ NanoDrop™ Lite Plus Microvolume UV Spectrophotometer can provide purity ratios and DNA concentrations for samples of just 1.0 – 2.0 µL volumes. The NanoDrop instrument's ability to analyze such small volumes and allow conservation of sample material for downstream experiments is critical, since most extractions elute or resuspend DNA in volumes below 50 µL.

Experimental Methods

Three samples of plasmid pUC19 DNA (Thermo Scientific, SD0061) were prepared. Sample 1 was pure pUC19 and samples 2 and 3 were pUC19 spiked with 150 ppm phenol and 20 mM EDTA, respectively, to mimic the contamination from a typical DNA extraction. The concentration, A260/A280 purity ratio, and A260/A230 purity ratio of all three samples were determined using 2.0 µL volumes on a NanoDrop Lite Plus spectrophotometer using the dsDNA sample type.

The contamination effect on restriction endonuclease cleavage was evaluated by digesting both pure and contaminated pUC19 with HindIII (Thermo Scientific, ER0501) and incubating for one and a half hours at 37°C. After incubation, HindIII was inactivated by heating at 80°C for 20 minutes. HindIII has one cleavage site in the pUC19 sequence, producing a linear plasmid of 2686 base pairs upon digestion (Figure 2).

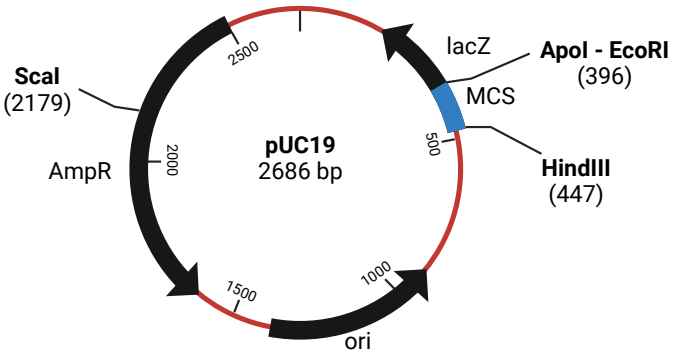

Figure 2. Plasmid map of pUC19 with several restriction endonuclease cleavage sites. Figure created with BioRender.com.

Gel electrophoresis of the digested pUC19 samples was performed with a 1.2% agarose gel to evaluate the endonuclease digestion efficiency and to confirm that the expected fragment length of 2686 bp was formed. The concentration of DNA loaded per well was equivalent to about 20 ng/µL. The bands on the gel corresponding to the digested plasmids were excised with a sterile scalpel. Excess gel was removed from each sample to yield 50 mg for downstream extraction.

Plasmid DNA was extracted from the three gel-excised samples by following manufacturer's instructions from the Invitrogen™ PureLink™ Quick Gel Extraction Kit (Invitrogen, K210012). Two minor changes were applied to the manufacturer's instructions to improve yield: 1) warming the elution buffer to 65°C before loading on the column; and 2) incubating the elution buffer on the column for 10 minutes before elution. The eluted DNA was analyzed for concentration and purity with the NanoDrop Lite Plus spectrophotometer using the dsDNA sample type.

	Concentration (ng/µL)	Standard Deviation (ng/µL)	A260/A280	A260/A230
Pure pUC19	241.8	0.6	1.90	2.03
pUC19 + Phenol (150 ppm)	358.3	0.5	1.77	1.76
pUC19 + EDTA (20 mM)	231	3	2.04	0.28

Table 1. Concentration and purity results of pure pUC19 and contaminated pUC19 measured in replicates of five on the NanoDrop Lite Plus spectrophotometer.

Results

The concentration and purity results of the pure and contaminated pUC19 samples determined by the NanoDrop Lite Plus instrument are outlined in Table 1. Pure pUC19 displayed an average concentration of 241.8 ng/µL and purity ratios in the expected range for dsDNA. Phenol contamination caused an increase in the reported concentration to 358.3 ng/µL due to the additional absorbance contribution of phenol at 260 nm.

The A260/A230 purity ratio of 1.76 fell below the expected range as phenol also contributes to increased absorbance below 230 nm. EDTA contamination increased the A260/A280 ratio and significantly reduced the A260/A230 ratio. Since salts are highly absorbing below 230 nm, contamination is clearly revealed in the A260/A230 purity ratio of 0.28.

When the purity ratios are outside of the expected range, the concentration should be considered unreliable due to the contamination effect on absorbance. At this stage, samples should be further purified with a column extraction or alcohol precipitation to ensure an accurate concentration measurement.

Figure 3 displays the gel electrophoresis results on the digestion efficiency of HindIII in the presence of EDTA and phenol contamination. Undigested pUC19 in column B remains in its supercoiled topology and travels further down the gel than its linear counterpart in column C.[6] The linear pUC19 control band was at the expected 2686 bp location in relation to the ladder in column A. Phenol contamination (column D) had a minimal effect on the digestion efficiency but higher concentrations of phenol or other organic solvents can further inhibit the restriction enzyme.[7-8] EDTA contamination inhibited HindIII digestion, as shown by the alignment of the band in column E with the undigested control in B. The results of the digestion confirm the importance of incorporating a purity checkpoint prior to running a restriction endonuclease digestion.

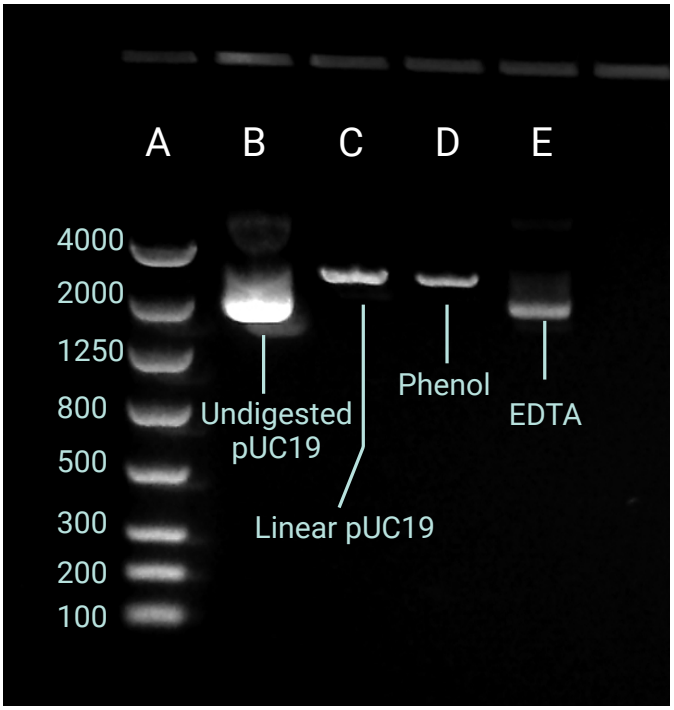

Figure 3. Gel electrophoresis image capture of supercoiled, undigested pUC19 (Lane B); linear pUC19 digested with HindIII (Lane C); pUC19 spiked with phenol (150 ppm) and HindIII digested (Lane D); pUC19 spiked with EDTA (20 mM) and HindIII digested (Lane E). Ran on a 1.2% agarose gel. Ladder in Lane A displayed as base pairs.

After performing a gel extraction of the three samples digested with HindIII, the purified samples were measured again on the NanoDrop Lite Plus spectrophotometer to calculate the sample concentration, percent recovery, and purity ratios. These results are presented in Table 2. The pure pUC19 sample was washed with wash buffer once while the phenol and EDTA contaminated samples were washed twice, which reduced the percent recovered DNA. The average concentration of recovered DNA from all samples was 15.04 ng/µL with a recovery of up to 89%. The A260/A280 and A260/A230 purity ratios for all samples were within the acceptable range except for the EDTA contaminated pUC19. With the reduced A260/A230 purity ratio, this is indicative of residual EDTA or guanidine salt from the extraction reagents. Incorporating isopropanol prior to loading on the extraction column aids in precipitating DNA away from salts and would lead to a purified DNA sample.[9]

Conclusions

The molecular cloning workflow requires QC checkpoints before and after the restriction endonuclease digestion to reduce the failure of downstream reactions. Contaminants such as phenol and salts have been shown to inhibit or reduce the efficiency of endonucleases, which highlights the need for performing a purity check before digestion. After digestion and gel extraction, purity should again be checked for ensuring successful ligation and transformation. With the NanoDrop Lite Plus spectrophotometer, the A260/A280 and A260/A230 purity ratios provide a fast and easy method for completing QC steps without the need for dilutions that require large volumes of extracted DNA.

	Concentration (ng/µL)	Standard Deviation (ng/µL)	% Recovery from Gel	A260/A280	A260/A230
Pure pUC19	17.83	0.09	89%	1.92	2.10
pUC19 + Phenol (150 ppm)	13.5	0.3	67.5%	1.84	2.04
pUC19 + EDTA (20 mM)	13.8	0.2	69%	1.95	1.69

Table 2. Concentration and purity results of gel extracted pUC19 measured in replicates of five on the NanoDrop Lite Plus spectrophotometer.

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