Assessing the Homogeneity of Plasmid DNA: An Important Step toward Gene Therapy

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Introduction

Plasmids are extra-chromosomal, double-stranded DNA molecules that may exist in several forms differing in topology and size. However, as plasmid DNA is being used as vectors for therapeutic genes, the development of good analytical processes to assess both purity and heterogeneity is of great importance.

With pharmaceutical-grade plasmid DNA, an adequate homogeneity of the final product is achieved when more than 90% of the molecules exist as the supercoiled covalently closed circular (ccc) form.1,2 This is the most compact form where the circular and covalently closed DNA helix is interwoven in itself, like a twisted rubber band. If one of the DNA strands is broken, the circular molecule relaxes under loss of coiling. This relaxed structure is called the open circular (oc) or nicked form. These topological plasmid structures may exist as different sizes, such as monomers and dimers, creating additional heterogeneity. Finally, linear plasmid structures are generated when both strands are cleaved at the same position.

Agarose gel electrophoresis (AGE) has been the primary method used to assess the homogeneity of plasmid DNA, but this approach has some major disadvantages. The AGE method is manual, only semi-quantitative, and the assignment of bands to plasmid structures is difficult since the electrophoretic mobility of plasmids of different shapes changes with the electrophoresis operating conditions.3,4 A more powerful routine technology for the quantification of plasmid forms is capillary gel electrophoresis (CGE).5 This automated approach offers high resolution, high sensitivity, and high reproducibility to this analysis. In this paper, we highlight a method allowing the separation and quantitation of all plasmid DNA forms (including oligomeric structures) typically present in a bacterial plasmid preparation.

Experimental

Capillary Electrophoresis

Analyses were performed using a P/ACE capillary electrophoresis system from Beckman Coulter equipped with a LIF detector (488/520 nm). Coated capillaries (DB-17; J&W Scientific, Folsom, CA, USA) with a length of 30 cm to the detector window, 100 μm I.D., and a coating thickness of 0.1 μm were used for separation. The capillary was flushed with run buffer (pH 8.4) consisting of 89 mM Tris, 89 mM boric acid, 2 mM EDTA, and 0.1% (w/w) hydroxypropylmethylcellulose (HPMC, Sigma, Deisenhofen, Germany). Just prior to analysis, the intercalating dye YOYO (Molecular Probes, Eugene, OR, USA) was added to the run buffer (1 μL YOYO in 15 mL run buffer). After pre-staining with YOYO at a DNA-base pair-to-dye molar ratio of 5:1, the plasmid samples were introduced hydrodynamically. Electrophoresis was carried out at 100 V/cm with the capillary thermostatted to 30°C.
Figure 1. Separation of pUC19 (2.7 kbp) plasmid structures.

Figure 2. Separation of pCMV-S2S (5.7 kbp) plasmid structures.

Figure 3. Linear correlation of corrected peak-area vs. DNA concentration of each plasmid structure of pCMV-S2S.

Figure 4. Electropherogram of typical plasmid prep.

Figure 5. Agarose gel electrophoresis: two untreated plasmid samples and one sample of the plasmid transferred into the oc-form.
**Plasmid Preparation**

pUC19 (2.7 kbp) and pCMV-S2S (5.7 kbp) plasmid DNA were isolated from overnight cultures of *Escherichia coli* using QIAGEN* Plasmid Maxi Kit (QIAGEN, Hilden, Germany). Linear plasmid DNA was prepared by digesting purified DNA with restriction endonuclease EcoRI (Roche Diagnostics, Mannheim, Germany). UV-treated plasmid DNA was irradiated with UV light resulting in single-strand breakage and relaxation of ccc to open circular structures.

**Results**

An electropherogram of a mixture of untreated, linearized, and UV-irradiated pUC19 plasmid DNA is presented in Figure 1. All monomeric and dimeric plasmid structures can be separated with baseline resolution. The order of migration is governed by the topology of plasmid structures. Supercoiled ccc molecules (monomers and dimers) have the most compact structure with the highest electrophoretic mobility—appearing earlier than linearized (monomers and dimers) forms that are followed by the open circular forms. This order of migration is further supported by the 5.7 kbp internal linear standard migrating more slowly than the linear pUC19 dimer of 5.4 kbp size.

When larger plasmids are analyzed, the same order of migration holds true. Figure 2 shows an electropherogram of a mixture of untreated, linearized, and UV-irradiated pCMV-S2S plasmid samples (5.7 kbp). The most compact ccc monomer and dimer structures migrate faster than linear and open circular structures. However, the resolution of oc monomers and dimers does appear to decrease with increasing plasmid size.

To accurately quantify the different forms of plasmid DNA, a linear correlation between the corrected peak area and the concentration of each structure should be demonstrated (as shown in Figure 3). In this example, an excellent linear correlation was achieved for the analysis of pCMV-S2S over a wide concentration range (0.06 to 4.0 mg/L). The supercoiling ratio of this plasmid sample was determined to be 86 ± 1.5% independent of the concentration applied. Figure 4 illustrates an electropherogram of a typical plasmid preparation, in comparison to the agarose gel image shown in Figure 5.

**Conclusions**

We have demonstrated the feasibility of using CGE as a quality control tool to assess the homogeneity of plasmid DNA for the purposes of gene therapy or genetic vaccination. This technique performs in a fast, automated, and highly reliable manner. The same order of migration, independent of plasmid size, is attained—simplifying the plasmid identification process.

This analytical method can be used as both a purity and heterogeneity assay during the cultivation of plasmid-bearing cells, during the purification of plasmid DNA, and during the formulation of plasmid-based therapeutics. This assay provides a basis for reliable stability studies and allows the establishment of quality assurance standards for plasmid DNA structural homogeneity.

**References**
