Long-Term Stability Study and Topology Analysis of Plasmid DNA By Capillary Gel Electrophoresis

M. Schleef, R. Baier, W. Walther, M.L. Michel, and M. Schmeer

The topology of plasmid DNA can be monitored with capillary gel electrophoresis (CGE) (**1**, **2**). An improved CGE technology was developed at PlasmidFactory to routinely determine the content of different plasmid forms that appear during cultivation and purification in DNA production runs. CGE is a useful tool for supporting process development in plasmid DNA and minicircle production, in drug delivery research, and in plasmid storage and stability evaluation studies.

Long-term storage of the *lac*Z (beta-galactosidase)–expressing pCMVß plasmid over a period of 13 months revealed that stable storage conditions at –80 °C (rather than storage at 4 °C) prevent an increase in open circular (*oc*) plasmid topology deriving from the degraded, supercoiled, covalently closed circular (*ccc*) form. Physical data were supported by in vivo gene transfer data, using jet-injection into different tumor models grown subcutaneously in mice. Additionally, plasmid pCMV-S2S produced for clinical applications has been stored at –20° C and monitored for more than five years with no detectable decrease of quality (no topology changes).

PRODUCT FOCUS: PLASMID DNA

PROCESS FOCUS: PRODUCTION, PURIFICATION, AND FORMULATIONS

WHO SHOULD READ: PROCESS DEVELOPMENT AND MANUFACTURING, QA/QC ANALYSTS, FORMULATION SCIENTISTS

KEYWORDS: GENE DELIVERY, STABILITY, LYOPHILIZATION, ELECTROPHORESIS

LEVEL: INTERMEDIATE

PLASMID TOPOLOGY

Plasmid DNA has been extended from a common tool for gene technology (e.g., transferring genes into eukaryotic cells) into use as an active pharmaceutical ingredient (API) for gene therapy and DNA vaccination. To fulfill regulatory requirements, plasmids for both research and therapeutics must be produced at the highest available purity and quality. Recently, gene medicine was heavily improved by novel technologies used to separate different plasmid topologies (**3**, **4**). Overviews describing use of plasmid DNA as pharmaceuticals were published earlier (**5**, **6**).

Typically, vector plasmids are supercoiled, double-stranded, circular DNA molecules between two and some 100,000 base pairs (kbp) in size (**7**). In addition to their monomer, covalently closed, circular (*ccc*) topology, DNA plasmids can appear in larger multimeric forms or in damaged structures, such as open circular (*oc*) or linear topologies. Single-strand breaks lead to the *oc* form, and double-strand breaks lead to the linear form. Both cause inactivation, especially if their damage is within the therapeutically active part of the construct.

SEBASTIAN KAULITZKI (WWW.ISTOCKPHOTO.COM)

Additionally, both single- and double-strand breaks lead to certain changes in the effective size of a (former) plasmid, which makes certain delivery methods (e.g., electrogene transfer) less effective (**8**, **9**).

Clearly defining a therapeutic gene product is necessary for determining the actually delivered form, particularly in clinical applications.

CGE ANALYSIS

Important developments were recently made in monitoring plasmid DNA drug formulations and improving quality assurance of such therapeutics (**10**): e.g., quantitation of potential contaminations such as RNA, genomic DNA, proteins, and lipopolysaccharides (endotoxins), as well as determination of homogeneity (percentage of the *ccc* form present). The different topologies of plasmid

DNA can be quantified by capillary gel electrophoresis (Figure 1). The order of migration depends on molecular topology and is — except in agarose gel electrophoresis (AGE) (**11**) — reproducible and always the same. Reproducibility is independent of plasmid size, thus simplifying detection and identification of CGE signals. In addition, CGE offers high resolution and sensitivity. The ratio of each plasmid topology present can be calculated by a standardized peak area (Figure 2), which correlates linearly to the DNA concentration for each plasmid topology (**10**).

In-process control (IPC) monitoring of plasmid topologies using CGE is important for verifying intact *ccc*-forms throughout a purification process — especially during steps in which strong shear forces affect DNA molecules (e.g., sterile filtration, chromatography, or formulation). Also, evaluation of biomass quality with respect to a plasmid product, and process monitoring of the necessary cell lysis procedure require quantification of the different topologies present in bacterial host cells (**12**).

DNA pharmaceutical formulation requires permanent control of plasmid topology to ensure an intact (and hence safe) and potent pharmaceutical ingredient based on plasmid DNA. CGE analysis has been performed also to analyze "decomplexed" DNA vectors with respect to their topology and for ensuring proper storage of the DNA for future applications.

Technical Set-Up

CGE analyses were performed using a P/ACE 2050 capillary gel electrophoresis system equipped with a 488–520 nm laser-induced fluorescence (LIF) detector, both from Beckman Coulter (www.beckmancoulter.com). We used DB-17 coated capillaries from J&W Scientific (now owned by Agilent Technologies, www.agilent.com) with an effective length of 30 cm to the detector window, an inner diameter of 100 µm, and a coating thickness of 0.1 µm, flushing each capillary with buffer solution just before analysis. Then we added the intercalating dye YOYO-1 from Molecular Probes (http://probes.invitrogen.com). After prestaining, plasmid samples were introduced hydrodynamically to the P/ACE system, and electrophoresis was carried out at 100 V/cm and 30 °C.

Figure 1: Electropherogram from CGE analyzing different plasmid topologies of pUC19 plasmid DNA (2.7 kb) shows the detected signal in relative fluorescence units (RFU) compared with migration time.

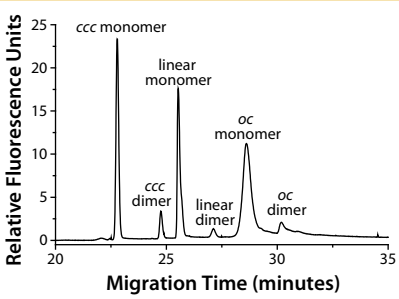
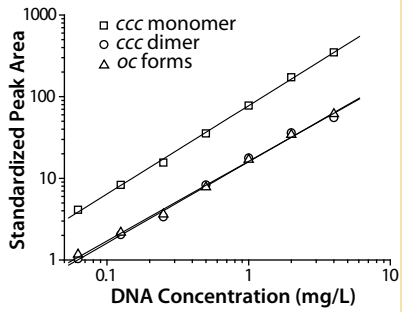


Figure 2: Linear correlation of standardized peak area (peak-area-to-migration-time ratio) for each plasmid structure



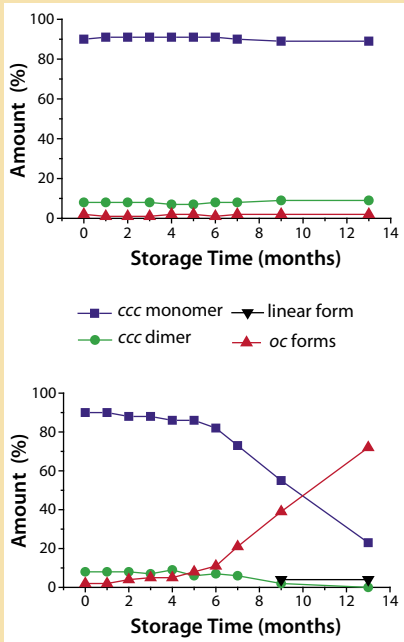
Long-Term Plasmid Vector Storage Analysis

Using CGE, the necessary physical stability of plasmid DNA for storage, shipment, and application can be monitored analyticaly. This tool quantifies relevant topological forms such as *ccc, oc,* and linear DNA and their respective dimer versions.

Storage at 4 °C does not alter plasmid topology significantly for several weeks. At temperatures lower than –20 °C, DNA storage is possible for years without significant loss of quality (Figure 3). Stability of plasmid DNA is associated with an effective in vivo gene transfer (**13**).

An alternative stabilization approach, used especially in vaccine formulations, is lyophilization. Freeze-drying technology is widely used for non-DNA biologics, such as monoclonal antibody and other protein formulations. Its application to plasmid DNA has not always been successful because reconstitution of freeze-dried plasmid DNA severely damaged its active *ccc* form. It is assumed that the reason for that is in some cases a too-compact lyophilizate that contains additional bacterial chromosomal DNA and other forms (such as *oc*) as well as lipids or proteins, which could lead to harsh conditions when resuspending the DNA "pellet." Recent developments in production have allowed more efficient purification of *ccc*-DNA (**3**), so the freeze-drying problem has been alleviated. Figure 4 shows CGE results based on the reconstitution of lyophilized plasmid DNA.

Figure 3: CGE was used for quantitative analysis of plasmid DNA isoforms. Plasmid samples were stored at –80 °C (TOP) or 4 °C (BOTTOM) over a period of up to 13 months before CGE analysis (**7**).



Further CGE Applications

Capillary gel electrophoresis is also a useful tool for evaluating potentially destructive influences of some gene delivery methods.

"Gene gun" jet-injection devices have been used in recent years for delivery of plasmid DNA therapeutics. However, they require further evaluation with respect to plasmid stability because associated shear

Figure 4: Comparing nonlyophilized (TOP) and lyophilized–reconstituted (BOTTOM) plasmid DNA indicates no difference in physical properties as shown by CGE (data from Schmidt et al. at PlasmidFactory).

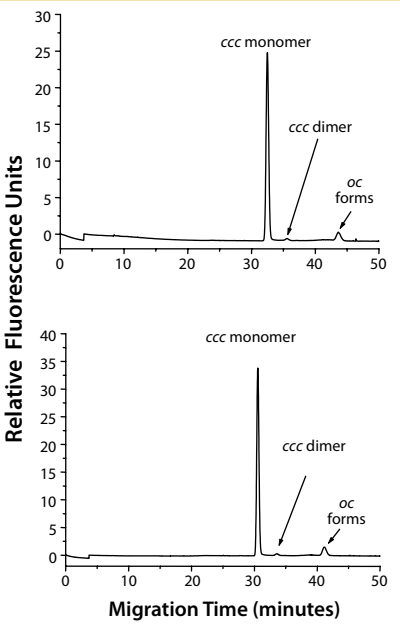
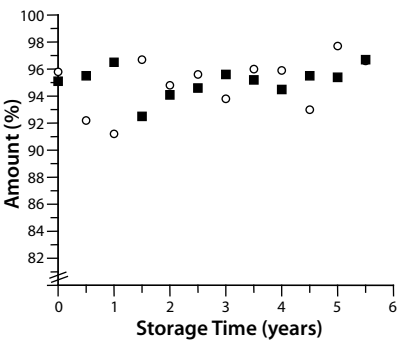


Figure 5: CGE was used for quantitative analysis of plasmid DNA (pCMV-S2S) topologies. Plasmid samples were stored at –20 °C for a period of over five years and then analyzed by CGE (squares) and AGE (circles). Data are presented as a percentage of each *ccc* plasmid form present in the analyzed samples.



forces could influence the molecular topology of plasmid pharmaceuticals.

Monitoring major changes from *ccc*- into *oc*- or linear topologies during gene-gun application is essential, as is optimizing injection pressure with respect to different tissues, plasmid sizes, buffers, and intended depth of injection. So far, CGE has appeared to be the only reliable and useful method for doing so (**14**).

The electroporation method is gaining increased importance in delivery of pharmaceutical DNA into specific tissues (**15**). We recently found that under certain conditions the associated high electric fields can significantly damage the *ccc* topology of a plasmid DNA, resulting in the presence of *oc* and linear forms (data not shown). That effect mainly occurs near the electrodes, perhaps due to brief local pH changes in the highly electrified region close to those electrode surfaces.

Clinical Applications

Use of plasmid DNA vectors for gene delivery in gene and cell therapy and for nucleic acid vaccination has required a tool such as CGE to determine and evaluate product quality during production (and lot release) and also in the moment of application with such nonviral vector systems. Usually weeks or months go by between production of a clinical sample and its release for clinical trials. To ensure the proper molecular topology, a sampling in parallel with application of the drug is necessary to determine the status of the pharmaceutical.

We performed routine CGE analysis for clinical plasmid DNA batches of pCMV-S2S carrying a hepatitis B virus vaccine (**16**), which had been stored at –20 °C for more than five years. Our analysis showed no major changes in vector topology, with a *ccc* fraction averaging 95% (Figure 5). The mean absolute deviation from that average value with agarose-gel electrophoresis (AGE) data measured in parallel was twice as high as the CGE mean. Therefore, CGE was found the more reliable technique.

Acknowledgments

The authors thank R. Merz and Dr. T. Schmidt for technical assistance and know-how in performing these CGE analyses.

References

1 Courtney BC, et al. Capillary Gel Electrophoresis As a Method to Determine Ligation Efficiency. *Anal. Biochem.* 228(2) 1995: 281–286.

2 Hammond RW, et al. Capillary Electrophoresis of Supercoiled and Linear DNA in Dilute Hydroxyethyl Cellulose Solution. *Anal. Chem.* 69(6) 1997: 1192–1196.

3 Schleef M, Schmidt T. Animal-Free Production of *ccc*-Supercoiled Plasmids for Research and Clinical Applications. *J. Gene Med.* 6(supplement 1) 2004: S45–53.

4 Molloy ML, et al. Effective and Robust Plasmid Topology Analysis and the Subsequent Characterization of the Plasmid Isoforms Thereby Observed. *Nucl. Acids Res.* 32(16) 2004: e129.

5 *Plasmids for Therapy and Vaccination.* Schleef M, Ed. Wiley-VCH: Weinheim, Germany, 2001.

6 *DNA Pharmaceuticals.* Schleef M, Ed. Wiley-VCH: Weinheim, Germany, 2005.

7 Schumann W. The Biology of Plasmids. *Plasmids for Therapy and Vaccination.* Schleef M, Ed. Wiley-VCH: Weinheim, Germany, 2001: 1–28.

8 Neumann E, et al. Mechanism of Electroporative Dye Uptake by Mouse B Cells. *Biophys. J.* 74(1) 1998: 98–108.

9 Maucksch C, Rudolph C. Department of Molecular Pneumology and Experimental Therapy Research, LMU (Munich, Germany) personal communication, 2006.

10 Schmidt T, et al. Quantitative Analysis of Plasmid Forms By Agarose and Capillary Gel Electrophoresis. *Anal. Biochem.* 274(2) 1999: 235–240.

11 Meyers JA, et al. Simple Agarose Gel Electrophoretic Method for the Identification and Characterization of Plasmid Deoxyribonucleic Acid. *J. Bacteriol.* 127(3) 1976: 1529–1537.

12 Schleef M, Voß C, Schmidt T. Novel Technology for Topological Plasmid Vaccine Characterization. *Nature Labscene* April 2002: 18–19.

13 Walther W, et al. Stability Analysis for Long-Term Storage of Naked DNA: Impact on Nonviral In Vivo Gene Transfer. *Anal. Biochem.* 318(2) 2003: 230–235.

14 Walther W, et al. Intratumoral Low Volume Jet-Injection for Efficient Non-Viral Gene Therapy. *Mol. Biotechnol.* 21(2) 2002: 105–115.

15 Mir LM, et al. Biomedical Applications of Electric Pulses with Special Emphasis on Antitumor Electrochemotherapy. *Bioelectrochem. Bioenerg.* 38(1) 1995: 203–207.

16 Michel M-L, et al. DNA-Mediated Immunization to the Hepatitis B Surface Antigen in Mice: Aspects of the Humoral Response Mimic Hepatitis B Viral Infection in Humans. *Proc. Natl. Acad. Sci. USA* 92(12) 1995: 5307–5311.

M. Schleef *is CEO,* ***R. Baier*** *is head of production, and* ***M. Schmeer*** *is product manager at PlasmidFactory GmbH & Co. KG, Meisenstr. 96, D-33607 Bielefeld, Germany.* ***W. Walther*** *is group leader at the Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Str. 10, D-13092 Berlin, Germany. And* ***M.L. Michel*** *is research director at INSERM, head of the department of virology at INSERM research unit U812, Institut Pasteur, 28 rue du Docteur Roux, F-75724 Paris Cedex 15, France.*