Effective and robust plasmid topology analysis and the subsequent characterization of the plasmid isoforms thereby observed

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ABSTRACT

Within the biopharmaceutical industry, recombinant plasmid DNA is used both as a raw material (e.g. in lentiviral and AAV vector production) as well as an active ingredient (e.g. in DNA vaccines). Consequently, many analytical laboratories are routinely involved with plasmid DNA topoisomerase qualitative analysis and quantification. In order to reliably determine plasmid topology, one must ensure that the methodology employed can reliably, precisely and accurately measure qualitatively and quantitatively all topological isoforms. Presented here are an anion-exchange high-performance liquid chromatography (AEC) and an agarose gel electrophoresis (AGE)-based method developed for this purpose. The strategies undertaken to overcome the respective typical problems of limited linear range of quantitation (for AGE) and isoform resolution (for AEC) are described. Also presented is a subsequent direct comparison (for assay precision/accuracy) of these two methods, as well as a package of species characterization [by chloroquine-AGE, enzymatic digestion, multi-angle laser light-scattering (MALLS) and electron microscopy] undertaken to confirm the identity of a minor supercoiled dimeric concatamer observed by both approaches.

INTRODUCTION

Because plasmid DNA can now be classed as either a biopharmaceutical raw material or an active ingredient [see (1) and (2) for respective examples], large-scale multi-gram batch preparation of plasmid DNA is commonplace (3). During such large-scale plasmid fermentation, plasmid molecules propagated within appropriate Escherichia coli host strains are maintained predominantly in a negatively supercoiled form by the host cell enzymes (4). However, after fermentation and during subsequent extraction and downstream processing, formulation and storage, a subset of these plasmid molecules will then become nicked on one strand to form the open-circle species or nicked on both strands (at or near the same junction) to form the linear species (5,6). As a consequence of such nicking, most purified plasmid batches will comprise a mixture of supercoiled, relaxed closed-circular, open-circular and linear monomeric species, as well as a minority of often less-defined, ‘other’ multimeric species and aggregates (7). Therefore, because plasmid nicking and the ensuing changes in topology are directly related to the process, formulation and storage conditions, determining the relative proportions of supercoiled, open-circle, linear and ‘other’ species in a given manufacturing batch provides reassurance about the batch process, formulation and storage consistency. It is for this reason that measuring topology profiles is now routinely performed within analytical laboratories.

Within our analytical development laboratories, two assays have been independently designed and developed with the aim of being able to reliably determine the relative abundance of the differing plasmid isoforms in a given plasmid preparation. One of these methods uses the well-established agarose gel electrophoresis (AGE) methodology to separate and then quantitate (by staining and subsequent image analysis) the differing topological forms, whilst the second exploits anion-exchange high-performance liquid chromatography (HPLC) (AEC) for the same purpose. Presented below is a detailed summary of the work undertaken to develop and evaluate these assays, as well as a formal comparison designed to assess the analytical capabilities of both assays, once developed. This direct assay comparison involved analysis of the same manufacturing batches of plasmids, the same stability study samples and the same spiked material by both approaches. Also presented are chloroquine-AGE, enzymatic digestion, multi-angle laser light scattering (MALLS) and electron microscopy (EM) analyses undertaken to confirm topological identity for a particular minor plasmid species routinely observed but never fully characterized previously by such a methodology.

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MATERIALS AND METHODS
Plasmid and plasmid manufacture
Plasmid extracts were prepared as described previously (3). The 6.5 kb ‘DNA vaccine’ plasmid analysed throughout the course of this study comprises a typical kanamycin resistance cassette and pUC origin of replication plus a mammalian expression cassette (pol II promoter/ORF/polyadenylation). Further details are available upon request.

Quantitative agarose electrophoresis
Horizontal gel electrophoresis was performed with 0.4 or 0.6% agarose gels prepared using 100 ml 1× TBE (Sigma–Aldrich, Poole, UK) and midigel apparatus (Owl, Portsmouth, NH) with 12-well combs. Electrophoresis was performed overnight (16–18 h) at 20 V. All staining was performed with 1× Sybr-Gold (Molecular Probes, Eugene, OR) made up in fresh 1× TBE and for the lengths of time indicated. Gel Image capture was by either a DOC-008 system (UVitec Cambridge, UK) or a ProXpress Proteomic Gel Imaging System (PerkinElmer, Cambridge, UK). For gel image quantification, the 16 bit depth images generated by the ProXpress were analysed using ImageQuant software version 5 (Molecular Dynamics, SunnyVale, CA).

Anion-exchange high-performance liquid chromatography
All AEC analyses were performed on a Waters 2690 separations module with a 2487 dual wavelength detector (Waters, Manchester, UK). Buffer A (borate buffer) was composed of 25 mM boric acid (Sigma–Aldrich) adjusted to pH 9.0 with 50% w/v sodium hydroxide (Sigma–Aldrich). Buffer B was composed of Buffer A with 1 M sodium chloride (Sigma–Aldrich). For all analyses, a 4.6 × 75 mm 2 Toso-Biosep DNA-NPR anion exchange column (Anachem, Cambridge, UK) with a Machery–Nagel pre-column filter (Machery–Nagel, Oxford, UK) was used, and the column oven temperature was set at 40°C. All plasmid DNA samples were prepared at 10 μg/ml in buffer A prior to 10 μl injections. The ultraviolet (UV) detection was at 260 nm with a flow rate of 1 ml/min and the gradients used were as described in Figure 2. For result analysis, Atlas 2000 version 4.30 (Thermo LabSystems Inc., Beverly, MA) was used.

Open-circle plasmid species generation
Open-circular topoisomers were prepared by incubating supercoiled plasmid DNA with the enzyme N.BstNB I (New England Biolabs, Beverly, MA) at a concentration of 1 U/μg. The reaction was carried out in 1× N.BstNB I buffer (supplied with the enzyme) at 55°C for 1 h. The enzyme was inactivated by the addition of EDTA (Sigma–Aldrich) to a final concentration of 0.025 M, followed by incubation at 80°C for 20 min. Digests were carried out such that the plasmid DNA in the final solution was at a concentration of 200 ng/μl. Successful digestion was confirmed by AGE.

Linear plasmid species generation
Linear plasmid preparations were generated by incubating supercoiled plasmid DNA with a single-cutting restriction enzyme (HindIII, New England Biolabs) in the buffer recommended by the manufacturer. The concentration of enzyme used was in excess of that suggested as necessary by the manufacturer. The reactions were incubated at 37°C for 2 h. The DNA was cleaned up by extraction with buffer-saturated phenol (Sigma–Aldrich) followed by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and then with chloroform (Sigma–Aldrich). Precipitation of the DNA was carried out by the addition of 0.1 volumes of 3 M sodium acetate and 2 volumes of ice-cold absolute ethanol (Sigma–Aldrich). After 20 min at −70°C, the DNA was pelleted by centrifugation. The pellet was washed with 70% ethanol and then re-suspended in water to give a final plasmid DNA concentration of 200 ng/μl, as confirmed by the absorbance at 260 nm. Successful linearization of the plasmid was confirmed by AGE.

Sample preparation for assay comparison
For the direct AEC and AGE comparative analysis, all samples were prepared at a concentration of 10 μg/ml in 25 mM borate buffer, pH 9.0 (AEC buffer A), so as to be compatible with both analytical approaches.

Fraction D topology analysis by T7 exonuclease enzyme digestion
To further characterize a particular plasmid species (fraction D) observed by both the AEC and AGE methodologies, the specificity of the T7 exonuclease was exploited. For this work, 1 μg of plasmid was incubated with T7 exonuclease (New England Biolabs) for 1 h, using the manufacturer’s recommended conditions, prior to AGE-based separation and analysis.

Fraction D analysis by chloroquine-AGE
A 25 × 20 cm 2 0.6% agarose (1× TBE) gel containing 5.0 μg/ml chloroquine (Sigma–Aldrich) was employed. The 1× TBE running buffer also contained 5.0 μg/ml chloroquine, and electrophoresis was performed at 50 V for 48 h. Staining was carried out by soaking in 1× TBE containing 1× Sybr-Gold (Molecular Probes) for 24 h in the dark.

Fraction D analysis by MALLS
For all MALLS analyses, a Wyatt Dawn EOS detector (Wyatt Technologies, Santa Barbara, CA) was connected in line to AEC (see above) and downstream of the UV detection.

RESULTS
Quantitative AGE assay development
The use of AGE for the separation of DNA topoisomers into discrete ‘bands’ has been considered routine for a number of decades [see (8) for an example]. These bands are then observed by gel staining with DNA intercalating agents such as ethidium bromide, prior to subsequent light excitation and film, video or digital image capture and analysis. However, whilst this simple method is excellent for both separating and distinguishing many plasmid species (supercoiled, relaxed, open-/closed-circular, linear, multimers and aggregates), it is far from ideal if information on the relative quantities of these differing species is then required. This is because the effective linear range of most, if not all, current
AGE gel staining and image capture methodologies is less than one order of magnitude (9). Consequently, if qualitative and quantitative data are required by AGE-based methodologies, it is normal to examine extensive serial dilutions of a test sample and then cross-reference this against an equally extensive serial dilution of a standard [e.g. see (10,11)]. Because of this problem, many analytical laboratories have preferred AEC and capillary electrophoresis (CE) separation methodologies for the analysis of a clinical grade plasmid material. However, the paradox of this preference is that these AEC and CE methodologies have not been chosen for their plasmid species separation capabilities, which can sometimes be inferior to AGE, but rather for their superior assay sensitivity and linear ranges of quantitation (M. J. Molloy, unpublished data). For these reasons, it was therefore decided to undertake a critical re-evaluation and re-development of an in-house AGE-based protocol in an attempt to improve assay sensitivity and linear range prior to the direct comparison with the AEC assay.

The in-house AGE protocol chosen for such re-development originally involved overnight separation of plasmid species by 0.6% AGE, subsequent staining with ethidium bromide, before final image capture with a video documentation system.

The first modification to this assay involved a replacement of the ethidium bromide-staining regime with a Sybr-Gold staining alternative. This unsymmetrical cyanine dye exhibits >1000-fold fluorescence enhancement upon binding nucleic acids and also has a high quantum yield upon such binding (12). Consequently, this switch to a staining reagent with such properties led to an immediate improvement in the assay by lowering the background noise and increasing the limit of detection. For comparison of typical images of an ethidium bromide-stained sample and the Sybr-Gold-stained equivalent see Figure 1A. This demonstrates that for plasmid preparations containing low-level recombinants, the Sybr-Gold-staining methodology is preferable.

Subsequent to this change in the staining reagent, the second assay modification involved an improvement to the quality of the gel image itself. This was undertaken in the belief that the relatively low quantitation range observed within AGE-based methods was primarily due to the relative low 8 bit image depth (2⁸ grey scales per pixel) (13) produced by the laboratory’s video documentation system. Consequently, by using, instead, a 16 bit (2¹⁶ grey scales per pixel) peltier-cooled (and thus low noise) charge-coupled device (CCD) imaging system, an increase in assay linear range would be expected. However, even after this change and even with careful image exposure trials, the assay linear range did not extend beyond 1–50 ng/band without the problem of a significant signal ‘plateau’ (see Figure 1B). As a consequence, and because the 16 bit gel images contained more than the adequate theoretical image depth, it was concluded that the image quality alone could not account for the low linear range of quantitation by the AGE-based methodology.

The third (and decisive) assay modification involved the Sybr-Gold staining regime. The manufacturer’s recommended staining time for Sybr-Gold is 20–40 min (12). However, by extending the staining times to well beyond this recommendation (up to a total of 24 h), it became apparent that a very good linear fit could be obtained to >300 ng/band loads and with no loss in sensitivity (see Figure 1C).

To conclude, as a consequence of changes to (i) the stain chosen, (ii) the image quality/depth and (iii) the staining time, the linear range of quantitation (per band) of an AGE-based assay was improved from ~5–50 to 0.5–300 ng. Consequently, all species in a 250 ng sample load could be reliably separated and quantified down to a limit of ~0.5 ng (0.2% total) and a limit of detection that is far less (0.1 ng or 0.04% total with a gel image over-exposure; data not shown).

AEC assay development

AEC exploits the interaction between the negatively charged phosphate groups on the plasmid DNA backbone and positively charged groups on the resin stationary phase. The overall interaction is thought not to be a distinct binding between specific charges, but rather a local attraction generated by opposite charges in close proximity (14). Therefore, and using a suitable salt gradient, this interaction is manipulated for effective resolution and quantitation of all differing topological isoforms present within a sample. This is possible because although the overall charge and molecular weight of differing topological species might be similar, these differing isoforms will possess different conformations and thus different local charge densities. Consequently, such species will possess differing column retention times in an increasing salt gradient.

Generally, for AEC-based analysis of DNA, method development requires optimizing the key parameters on a case-by-case basis. To this end, several groups have evaluated different anion exchange resins, mobile phase components, flow rate, pH, column temperature and gradient slopes (15–17). The initial in-house method adopted here utilized a 20 mM Tris-based buffer (pH 9), a relatively shallow salt gradient (1% Buffer B/min), a non-porous resin and a thermostat-controlled Waters 2690 column oven which allows for mobile phase pre-heating [so as to avoid unwanted complications (18)]. However, because of the retention time drift and the subsequent isoform resolution issues with this initial method (data not shown), further development was needed. This development involved changing the buffer base (from 20 mM Tris to 25 mM borate) and then subsequent re-optimization of the eluting salt gradient. The buffer was changed for two reasons. First, at pH 9 a Tris-based buffer is close to the limit of its buffering capacity (pKa of Tris = 8.06) and previous in-house work suggests that a pH of 9 is optimal for plasmid isoform resolution (M. J. Molloy, unpublished data). Second, Tris buffers possess relatively high δpKa/δT values (−0.028°C⁻¹) when compared with borate (−0.008°C⁻¹) value) (19) and so may induce significant pH buffer changes in any temperature gradient between the 40°C and the ambient buffer. After changing the buffer, re-optimization of the elution gradient was then undertaken as outlined in Figure 2. The results suggest that a salt gradient with a slope of 4% buffer B per minute was most suitable (with regard to resolution and peak tail factors) on our in-house AEC system. Consequently, for the analysis of the 6.5 kb plasmid used in this AEC assay optimization, a 25 mM borate buffer and a 4% gradient is optimal. For all subsequent AEC/AGE direct assay comparison (as will now be described), this same 6.5 kb plasmid (or linear, nicked, spiked or degraded derivatives thereof) has been used on all occasions.
HPLC and Q-AGE direct comparison

After the completion of AGE and AEC assay development, a direct assay comparison was initiated. This comparison involved a systematic analysis of the same samples by both methodologies, and the preparation of the samples chosen for this analysis is described within Materials and Methods. In brief, this comparison was initially composed of two component parts. The first component involved analysis of purified plasmid or its temperature-degraded equivalent (stored at 37°C for 18 days prior to re-freezing) in triplicate on three separate occasions by both methods. For the second component, an analysis of 18 stability study time-points by both methods was undertaken. As a consequence of this assay comparison, a third component was then added. This involved analysis of equivalent amounts of near-pure supercoiled, linear and open-circle species by both methodologies.

The results of this work are outlined in Figures 3–5 and demonstrate that both methodologies are acceptably precise (see RSD values, Figure 3D) and that both report broadly equivalent results. However, also apparent is the fact that both qualitative and quantitative differences do exist between the two assays. These differences will now be summarized.

Quantitative differences

The most notable quantitative difference between the two assays relates to the levels of open-circular DNA reported.
Figure 2. AEC assay development. Representative analytical traces generated by analysis of plasmid DNA using 2, 3, 4 and 5% gradients per minute as indicated (from left to right, respectively). Details of Buffer A (25 mM borate) and Buffer B (25 mM borate, 1 M NaCl) mixing parameters over time (in minutes) for each gradient are also included in the associated table. The flow rate (1 ml/min) and the column temperature (40°C) were constant for all analyses. For all gradients, the order of elution from open-circle (OC, first off the column) through to fraction D (D, last off) was maintained. However, note that for a 2% gradient, complete separation of supercoiled (SC), linear (L) and fraction D (D) was not possible. The retention times of all known peaks correlated with the enzymatically prepared reference standards (data not shown). For characterization of the unknown peak see Figures 5 and 6 (fraction D).

<table>
<thead>
<tr>
<th>Gradient</th>
<th>2%</th>
<th>3%</th>
<th>4%</th>
<th>5%</th>
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<td>Time (mins) to End Conc. (1ml/min flow)</td>
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<td>7</td>
<td>5</td>
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<td>55%</td>
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<tr>
<td>End Conc. Buffer B</td>
<td>75%</td>
<td>76%</td>
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Figure 3. Results from a Q-AGE and AEC direct assay comparison comprising two samples analysed in triplicate on three separate occasions. (A) Example gel image obtained by AGE (by 24 h Sybr-Gold staining methodology). Triplet 200 ng loads of purified plasmid (lanes 1–3) and 37°C stored purified plasmid (lanes 4–6) were analysed. The position of the supercoiled (SC), linear (L) and open-circle (OC) species are indicated. The schematic longitudinal tracks through samples 1 and 4 are a representation of the cross sections subsequently analysed by ImageQuant. (B) ImageQuant generated schematic traces obtained from the cross sections of lanes 1 and 4 seen by AGE. (C) The corresponding AEC schematic traces obtained from the two different samples. (D) Tabulated results of AGE and AEC direct comparison. For each sample type (purified plasmid or temperature-induced degraded plasmid), the values for the mean and relative standard deviation (RSD) of triplicate runs on three separate occasions are presented (i.e. nine values per mean result). The RSD value obtained (in parentheses) can be considered representative of total intra-assay precision (repeatability).
It was noted both here and by previous work (data not shown), that the AEC assay reported lower levels relative to AGE. Indeed, it was because of such observations that the third component part of the comparison was undertaken. In this subsequent third investigation, identical aliquots of supercoiled plasmids were treated with (i) BSA (control), (ii) a linear generating restriction enzyme or (iii) a nicking (open-circle generating) enzyme. After these differing treatments, equal amounts of all three were analysed, without subsequent purification steps. As a consequence of this work, and because without subsequent post-reaction purification and re-quantitation, equivalent loads of DNA should still be present, it was of interest to determine whether equivalent total sample load ‘area-under-the-curve’ values were seen for the three differing preparations by both methodologies. The results of this work are represented in Figure 5A and demonstrate that whilst by Q-AGE equivalent amounts were present, by AEC, open-circle levels were reported on average at only 54%—the values seen for the supercoiled or linear species. Therefore, and because subsequent nucleoside content analysis of the linear, open-circle and supercoiled preparations (by enzymatic digestion and AEC analysis) suggested that there was indeed equivalent levels of DNA in all three samples (data not shown), it was concluded that open-circle levels are under-reported by the AEC approach. Finally, it was also noted that because equivalent amounts of the three isoforms generate equivalent fluorescent signals by 24 h staining regimes, from a practical perspective, there appears to be little bias in Sybr-Gold binding affinity for the differing plasmid topoisomers. However, further work is still required before this conclusion can be extended and quoted for free-solution binding studies. This is because for ethidium bromide, an apparent similar lack of bias sometimes observed within gel-based staining regimes (20,21) is not replicated within classic free-solution assays (22).

For further emphasis of the difference in open-circle-level reporting by the two methods, an AEC ‘dedicated’ 10% linear/10% open-circle spiked supercoiled sample was then generated prior to subsequent analysis by AGE. The results of this analysis (see Figure 5B) reveal this spiked sample to report open-circle content at 7.9 and 22% for AEC and AGE methods, respectively. Additionally, the observation that for

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**Figure 4.** A direct assay comparison: Stability study samples analysed by AGE- and HPLC-based methodologies. (A) Gel image of the −20, +4 or +37°C stored samples separated by 0.6% AGE. Lane order: −70°C stored purified plasmid reference standard (ref), time zero (lane 0), 1 month time point (lane 1), 2 month time point (lane 2), 3 month time point (lane 3), 4 month time point (lane 4), 5 month time point (lane 5), 6 month time point (lane 6). The positions of the supercoiled (SC), linear (L) and open-circle species (OC) are indicated. (B) Line plot of stability study results as analysed by AGE methodology. Relative proportions of each topological sub-species (x-axis) over time (y-axis) are presented. Also included are numerical results at the final 6 month time point (in parentheses). (C) Line graph plot of stability study results as analysed by HPLC methodology. Relative proportion of each topological sub-species (x-axis) over time (y-axis) are presented. Also included are numerical results at the final 6 month time point (in parentheses).
all samples the supercoiled/linear signal ratio is equivalent when analysed by both assays (ongoing observation; data not shown) further supports the conclusion that this is an open-circle species-related issue.

### Qualitative differences and fraction D characterization

The most striking qualitative difference between the two methods of plasmid analysis is that of the appearance of a distinct peak by AEC (termed fraction D) that was first observed during the AEC assay optimization (Figure 2). This species initially appeared to have no equivalent via the AGE assay. Therefore, in order to investigate this fraction further, it was collected, purified by AEC and then re-analysed by AGE. This re-analysis revealed it to migrate at a position very close to the open-circle species (Figure 6A) and so initially suggested that it may be a form of relaxed-circular DNA that one can separate via charge–density/size (AEC) but not via shape/size (AGE) parameters. However, further work then revealed that this fraction could be separated as a discrete and separate band by AGE, provided that a lower percentage (0.4%) gel was employed (Figure 6B). This appearance (and then the disappearance) of this species during a linearizing restriction enzyme digest suggests that this fraction contains a 13 kb concatamer species containing two enzymatic sites, rather than an interwoven catenane of two plasmids each with one such site. Subsequent EM analysis suggests that all fractions contain the expected species (Figure 7A). In addition, relative size and weight analysis of these fractions by MALLS confirmed fraction D to be nearly twice the molecular weight (see Figure 7B) of the other three fractions.

Finally, and with regard to the difficulties of fraction D resolution by 0.6% AGE, further work revealed that this was due to a peculiarity of plasmid size. This was demonstrated by the analysis of smaller insert derivatives of the same plasmid (Figure 6D) that revealed fraction D and open-circle species to be more readily resolved when smaller plasmids are analysed. This open-circle/concatamer co-migration under certain conditions is also supported by previous observations (24).

### DISCUSSION

Whilst qualitative measurement of plasmid topology profiles is commonplace (particular by AGE), till date, little true...
consideration has been presented with regard to understanding topology assays along the classic analytical guidelines centred on the limit of detection, limit of quantitation, linearity, precision, accuracy, range and specificity. It is for such reasons that we undertook the assay development and evaluation work described previously. Initially, three approaches (CE, AGE and AEC) were considered. However, due to the poor assay robustness observed for our current in-house CE approach (data not shown), the separated, differently linked forms observed are of low image intensity. Consequently, the work presented here describing the use of 16 bit CCD imaging equipment, appropriate dyes and unique, extended end-point staining protocols is expected to be of benefit not only to AGE-based plasmid topology assays but also to AGE-based quantitative analysis of DNA in general. For example, because the assay linear range extends from <0.5 ng to >300 ng (generating a $2^{-1}$ to $2^{8}$ ng quantitation ‘window’), effective relative quantification of PCR amplicons should be more easily achieved. This would, thereby, reduce laboratory demands on CE (25) and ‘real-time’ PCR (26) analysis as it should allow effective AGE-based PCR amplicon quantitation and qualitative analysis without excessive serial dilution. Furthermore, with the use of wider gel combs (to prevent ‘smearly’ over-loading (data not shown)) plus longer, more concentrated staining regimes, it is expected that this linear range could improve still further if the need arose.

For the AGE-based approach, assay development focussed on improving the assay linear range of quantitation because no simple approach to overcome this assay limitation has been reported previously. Consequently, the work presented here describing the use of 16 bit CCD imaging equipment, appropriate dyes and unique, extended end-point staining protocols is expected to be of benefit not only to AGE-based plasmid topology assays but also to AGE-based quantitative analysis of DNA in general. For example, because the assay linear range extends from <0.5 ng to >300 ng (generating a $2^{-1}$ to $2^{8}$ ng quantitation ‘window’), effective relative quantification of PCR amplicons should be more easily achieved. This would, thereby, reduce laboratory demands on CE (25) and ‘real-time’ PCR (26) analysis as it should allow effective AGE-based PCR amplicon quantitation and qualitative analysis without excessive serial dilution. Furthermore, with the use of wider gel combs (to prevent ‘smearly’ over-loading (data not shown)) plus longer, more concentrated staining regimes, it is expected that this linear range could improve still further if the need arose.

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For AEC-based topology analysis, the typical analytical issues are not centred on the linear range of quantitation but, instead, on the isoform specificity because without the correct separation methodology, the differing topological forms will often possess overlapping retention times. Therefore, and in an attempt to overcome this problem, we have investigated the parameters involved with AEC topology assay development and have concluded that the gradient slope and
the buffer used are both critical factors. However, because our in-house AEC assay remains bespoke for one particular plasmid (data not shown), the AEC methodology presented cannot be considered generic.

The subsequent direct comparison of the AEC and AGE assays developed revealed that both quantitative assays were acceptably precise. However, due to the nature of topology reporting (relative quantitation of all species within a single load), many variables introduced between replicate analysis (e.g. from pipette errors) will be removed and, as such, good precision is expected (except where the assay noise becomes a significant component of the signal). With respect to the assay accuracy, it was still concluded that the open-circle species are under-reported by AEC even though this is partly attributable to the fact that AEC resolves fraction D and the open-circle DNA as separate entities. This conclusion was made because of subsequent work (captured in Figure 5) which demonstrates a ~50% under-reporting of open-circle species by AEC. Subsequent further investigation into the AEC method suggests that up to the point of column saturation, a proportion of such species remain bound even during elution in high salt (data not shown). If such findings are reproducible, it should help explain this situation.

These observations, combined with the fact that the AGE-based approach is both generic and also readily capable of resolving high molecular weight aggregates and permanently denatured plasmid (data not shown), suggest that the AGE approach might be more suitable for most applications, provided fraction D/open-circle co-migration is carefully considered. Even so, and because in many ways AGE and AEC are complementary, it is likely that both will be employed for future in-house plasmid characterization. Consequently, we are continuing to develop the two methodologies further with focus on the appropriate assay system suitability tests [peak-to-peak resolution factors, retention times, column efficiency (plate value) and peak tail factor] for AEC, and the use of suitably compliant imaging kit and secure data analysis software for the AGE approach.

With regard to characterization of fraction D, whilst this species is often observed in plasmid preparations, it has rarely

![Figure 7](image-url)
been characterized further. As a consequence, and because minor levels of this species are observed within many plasmids of differing ‘backbones’ (data not shown), it is hoped that such complete characterization will be of interest to the fields of both pure and applied plasmid research.

In conclusion, a combination of effective assay development and the use of appropriate orthogonal and complementary approaches as those described herein (AEC, AGE, chloroquin-AGE, MALLS, EM and enzyme digestion) should allow for near-complete resolution, qualitative measurement and quantification of all plasmid classes within any given plasmid preparation. Thus, whilst the in-house batch-to-batch manufacturing topology profile consistency is both preferable and expected, if variations do occur (e.g. during changes in fermentation, downstream processing, formulation or storage), these will be accurately, precisely and sensitively detected, and then quantified.

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