

**Plasmid DNA Manufacturing: CMC and Regulatory Challenges for Advanced Therapeutic Medicinal Products (ATMPs)****Krunal Kothari<sup>1</sup>, Smit Nayak<sup>2</sup>, Pooja Dave<sup>3</sup>, Nishant Madhukar<sup>4</sup>, Drasti Patel<sup>5</sup>, Saumil Shah<sup>6</sup>**<sup>1</sup>Manager, Regulatory Affairs – CMC, Vertex Pharmaceuticals, Boston, MA<sup>2</sup>Senior Specialist, Quality Assurance – Moderna Therapeutics, Boston, MA<sup>3</sup>Assistant Professor, Department of Pharmaceutics, Parul Institute of Technology & Research, Parul University Vadodara, Gujarat, India<sup>4</sup>Regulatory Affairs Specialist, Baxter, Milwaukee WI<sup>5</sup>Research Scholar, Government Pharmacy college, Gandhinagar, Gujarat- 382028<sup>6</sup>Principal Associate Scientist, bluebird bio, Charlestown- MA

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**Abstract:**

Plasmid DNA is important for producing advanced therapy medicinal products (ATMPs) such as gene therapies, viral vectors, mRNA vaccines and engineered cellular therapies. Global demand has increased clinical and commercial manufacturing requirements for scalable, high-quality and regulatory-compliant pDNA manufacture. This review focuses on current pDNA manufacture strategies by evaluating upstream (host strain engineering, copy number optimization and high cell density fermentation) and downstream (alkaline lysis, chromatographic purification, and ultrafiltration/diafiltration) systems. It also discusses regulatory expectations from the FDA, EMA and ICH, particularly concerning process qualification, comparability, and lifecycle. There are challenges to ensuring pDNA is scalable, retains the desired topology and is free from impurities, which need resolution. In addition, novel manufacturing processes such as continuous processing, single-use technologies, cell-free DNA synthesis and artificial intelligence process monitoring may positively impact pDNA manufacture efficiency and quality. Collectively, these advances will help to shape the future of plasmid DNA biomanufacturing while providing ongoing support and facilitating the continued development of advanced therapeutic products.

**Keywords:** Plasmid DNA, Gene Therapy, Biomanufacturing, Critical Quality Attributes, GMP, Regulatory Affairs, Advanced Therapeutics.

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**Introduction**

Plasmid DNA is a circular double-stranded DNA molecule used extensively as a starting material for advanced therapeutics, including viral vectors (AAV, lentivirus), mRNA vaccines, and cell-based therapies (e.g., CAR-T, TCR-modified T cells) [1,2]. Unlike conventional biologics, pDNA serves as a template for transcription or transfection rather than as the therapeutic itself, yet its quality directly impacts safety, potency, and consistency of the final product [3].

The Chemistry, Manufacturing, and Controls (CMC) framework for plasmid DNA requires control of critical quality attributes (CQAs), including supercoiled content, residual genomic DNA, endotoxin levels, RNA/protein impurities, and sequence integrity [4]. Regulatory agencies, such as the FDA (CBER), EMA (ATMP unit), and PMDA, have issued expectations that highlight product characterization, process validation, and lifecycle man-

agement [5,6]. The ICH Q5A(R2) (viral safety), Q6B (specifications), and Q12 (post-approval changes) provide harmonized guidance applicable to plasmid-based products [7,8]. This review provides a detailed analysis of plasmid DNA manufacturing strategies and regulatory considerations, with emphasis on process complexity, analytical modernization, scalability challenges, and emerging trends.

**Methodology**

A structured and integrative literature review was conducted to systematically evaluate manufacturing strategies and regulatory considerations for plasmid DNA (pDNA) used in advanced therapeutics, including gene therapies, mRNA vaccines, and cell-based therapies.

**Literature Sources and Data Collection:** A comprehensive search of peer-reviewed scientific litera-

ture was performed using electronic databases including PubMed, Scopus, and Google Scholar.

In addition, primary regulatory documents and guidance were obtained from official sources, including:

- U.S. Food and Drug Administration (FDA) guidance documents on plasmid DNA and gene therapy products
- European Medicines Agency (EMA) guidelines related to advanced therapy medicinal products (ATMPs)
- International Council for Harmonisation (ICH) guidelines, including Q5A(R2), Q6B, and Q12

These sources were prioritized to ensure alignment with current global regulatory expectations and industry practices.

### Search Strategy

The literature search was conducted using predefined keywords combined with Boolean operators (AND, OR) to ensure comprehensive coverage. Key search terms included:

- “plasmid DNA manufacturing” AND “bioprocessing”
- “pDNA purification” AND “chromatography”
- “anion exchange chromatography” OR “hydrophobic interaction chromatography”
- “supercoiled DNA” AND “quality attributes”
- “CMC plasmid DNA” AND “regulatory guidelines”
- “FDA gene therapy CMC” OR “EMA ATMP guidelines”

### Inclusion Criteria

Publications and documents were included based on the following criteria:

- Published between [January-2015](#) and [September-2025](#), with inclusion of foundational studies where necessary
- Peer-reviewed journal articles, regulatory guidelines, and authoritative industry reports
- Studies focusing on plasmid DNA manufacturing, purification, CQAs, or regulatory frameworks

### Exclusion Criteria

The following sources were excluded:

- Non-English publications
- Studies not directly relevant to plasmid DNA or advanced therapeutics

- **Outdated literature not aligned with current regulatory or technological practices**

### Data Extraction and Thematic Analysis

Relevant information from selected sources was systematically extracted and categorized into key thematic domains aligned with the objectives of this review:

- Upstream manufacturing strategies (host strain engineering, fermentation, copy number control)
- Downstream purification techniques (alkaline lysis, chromatographic separation, UF/DF)
- Critical Quality Attributes (CQAs) (supercoiled content, impurity clearance, scalability)
- Regulatory frameworks (FDA, EMA, ICH guidelines)
- Process control and validation approaches (QbD, PAT, comparability studies)
- Emerging technologies (continuous manufacturing, single-use systems, cell-free DNA synthesis, AI-driven monitoring)

A comparative and integrative approach was used to identify key trends, regulatory expectations, and technological advancements across the field.

### Critical Appraisal and Synthesis

The selected literature was critically evaluated for scientific robustness, regulatory relevance, and applicability to real-world biomanufacturing. Emphasis was placed on:

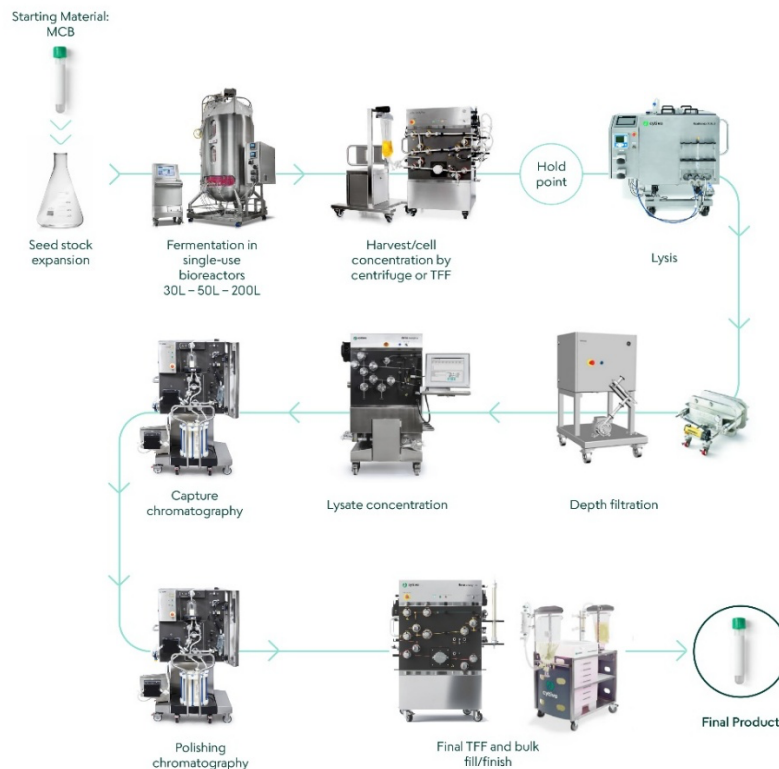
- Consistency with global regulatory expectations
- Strength of experimental and analytical methodologies
- Relevance to large-scale manufacturing and commercialization

Findings were synthesized to provide a regulatory-science-driven perspective, integrating both established practices and emerging innovations in plasmid DNA manufacturing.

### Limitations of the Review

This review is limited by reliance on publicly available literature and regulatory documents. Proprietary industry data and confidential regulatory submissions were not accessible. Additionally, rapid advancements in plasmid DNA manufacturing technologies and evolving regulatory expectations may extend beyond the scope of the reviewed literature

### Manufacturing Strategies for Plasmid DNA



**Figure 1 Plasmid facility and quality systems [49]**

Plasmid DNA (pDNA) has become a cornerstone raw material in the production of advanced therapy medicinal products (ATMPs), particularly viral vectors, CAR-T cell therapies, mRNA vaccines, and genome-editing platforms [9-11]. The unique physicochemical properties of pDNA—circular topology, supercoiled conformation, and large molecular weight—demand carefully designed upstream and downstream manufacturing strategies that differ substantially from conventional protein biologics [12,13].

### Upstream Manufacturing

- **Host Strain Selection and Engineering**

Most GMP-grade plasmids are produced in *Escherichia coli*, a well-characterized host with decades of industrial use [14]. Early strains such as DH5 $\alpha$  and JM109 remain common, but engineered strains optimized for high copy number and reduced recombination are increasingly preferred [15]. Recently, antibiotic-free systems such as operator-repressor titration (ORT), toxin-antitoxin systems, and auxotrophic complementation have replaced resistance markers to meet regulatory concerns over antibiotic resistance dissemination [16,17]. Strain selection also directly influences yield, supercoiling efficiency, and endotoxin burden.

- **Fermentation and Scale-Up**

High-cell-density fed-batch fermentation is the dominant strategy for pDNA production, typically

reaching biomass densities of 50–100 g/L dry cell weight [10]. Controlled feeding of glucose or glycerol prevents acetate accumulation, which can reduce plasmid yield and quality [18]. Temperature shifts (e.g., 37 °C to 30 °C post-induction) may stabilize supercoiled forms [19]. Emerging continuous and perfusion fermentations show promise for high-volume supply, although scale-up reproducibility and process monitoring remain challenges [20].

- **Copy Number and Stability Control**

Plasmid replication is controlled by origins of replication (ColE1, pUC, pMB1, etc.), which dictate copy number and influence downstream purification loads [21]. Over-replication can increase yield but also elevates impurities such as host DNA and RNA [15]. Vector design—including optimized promoters, terminators, and reduced homology regions—mitigates recombination and enhances sequence integrity [22].

### Downstream Purification

- **Cell Lysis and Clarification**

Alkaline lysis remains the gold standard, using NaOH/SDS to disrupt cell walls and release plasmid DNA, followed by neutralization with potassium acetate [23]. The resulting lysate contains plasmid DNA, host genomic DNA, RNA, proteins, and lipopolysaccharides (endotoxins). Clarification

is achieved via depth filtration, centrifugation, or flocculants [24].

## Plasmid Purification – Contaminant Removal

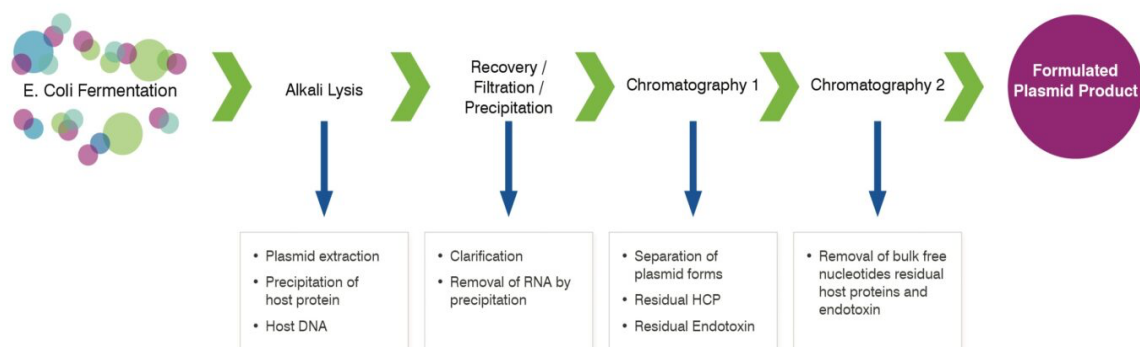


Figure 2 Phase dependent approach to plasmid manufacturing [50]

### • Chromatographic Separation

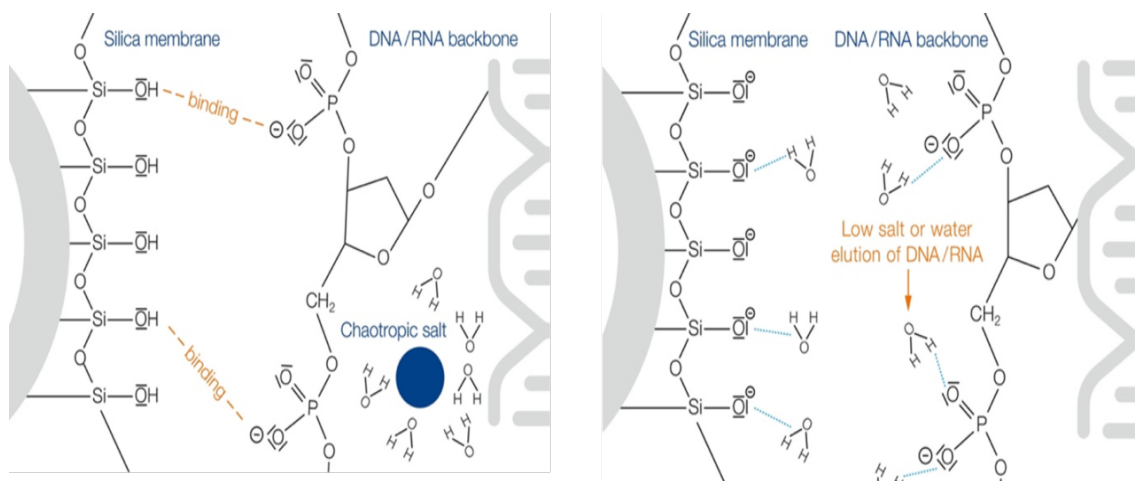


Figure 3 Anion Exchange Chromatography [51]

Chromatographic purification is a cornerstone of plasmid DNA (pDNA) manufacturing, enabling the efficient removal of process- and product-related impurities while enriching the supercoiled (SC) isoform—the therapeutically active conformation required for gene therapy and vaccine applications. Among the available techniques, Anion Exchange Chromatography (AEX) remains the workhorse for primary capture and impurity clearance. This method exploits the negatively charged phosphate backbone of pDNA to achieve selective binding and separation from host cell proteins, RNA, and endotoxins, typically utilizing quaternary amine ligands such as Q-Sepharose or DEAE-based resins [25,29]. Optimized salt gradient elution enhances recovery and purity of the SC fraction.

Hydrophobic Interaction Chromatography (HIC) serves as a powerful intermediate step to resolve topological isoforms—specifically discriminating between supercoiled, open circular, and linear

plasmids—based on differences in surface hydrophobicity. HIC is particularly valuable for refining SC content above 95%, which is essential for therapeutic-grade pDNA and regulatory compliance [26,30].

Size Exclusion Chromatography (SEC) is commonly employed as a polishing step for the removal of aggregates, multimers, and residual small-molecule contaminants such as salts and nucleotides. The technique provides a mild, non-denaturing environment that preserves plasmid integrity, often following AEX or HIC to ensure high final product homogeneity [27,31].

In recent years, membrane chromatography has emerged as an attractive alternative to resin-based systems, offering advantages in scalability, reduced buffer consumption, and operational cost efficiency. Membrane-based AEX and HIC modules provide high throughput under convective mass transfer, making them particularly suitable for large-

scale manufacturing of plasmids for vaccines and advanced therapeutic medicinal products (ATMPs) [28,32,33]. The shift toward single-use membrane platforms also aligns with the industry's transition to modular and continuous manufacturing systems, reducing cross-contamination risk and simplifying cleaning validation.

- **Polishing and Concentration**

Final steps include ultrafiltration/diafiltration (UF/DF) to concentrate plasmid DNA and exchange into formulation buffer [29]. Endotoxin removal is essential, with acceptable GMP limits typically <5 EU/mg plasmid DNA [30]. Detergent-based phase separation and specialized affinity resins have been introduced to enhance endotoxin clearance [31].

### 3. Critical Quality Attributes (CQAs) and Process Control

- **Supercoiled Content**

The supercoiled (SC) isoform represents the therapeutically active configuration of plasmid DNA, offering superior transfection efficiency and higher transgene expression relative to open circular (OC) or linear forms. Regulatory authorities—including the FDA and EMA—typically require a minimum of 80% SC content for clinical-grade plasmid preparations [32]. Maintaining this ratio demands stringent process control across all manufacturing stages. Excessive shear during fermentation, alkaline lysis, or chromatographic purification can induce nicking or strand breaks, converting SC DNA into OC or linear forms. Process optimization—such as low-shear impeller designs, controlled mixing rates, and buffer systems with stabilizing agents (e.g., EDTA, NaCl)—is therefore critical. In addition, enzymatic degradation by host nucleases must be minimized through rapid neutralization and temperature control during lysis and clarification [41].

- **Impurity Clearance**

Robust impurity clearance is a key quality and safety determinant for plasmid DNA intended for in vivo gene delivery or vaccine use. Regulatory expectations, as outlined by the World Health Organization (WHO) and FDA CMC guidelines for DNA vaccines, specify stringent impurity limits—typically <10 ng of residual host-cell DNA per dose and <100 pg of host-cell protein per  $\mu\text{g}$  plasmid [33,42]. RNA impurities, which can co-purify with pDNA due to similar physicochemical properties, are removed using RNase treatment and optimized chromatographic selectivity. The endotoxin burden (lipopolysaccharide from *E. coli* outer membranes) must also be reduced to <5 EU/mL, as it poses significant pyrogenicity and immunogenicity risks [34,43]. Comprehensive impurity control

is achieved through a combination of selective precipitation, enzymatic digestion, and orthogonal chromatographic steps, followed by validated analytical methods such as qPCR for host DNA and LAL assay for endotoxin quantification.

- **Scalability and Reproducibility**

A critical regulatory requirement for plasmid DNA manufacturing is the demonstration of consistent yield, purity, and CQAs across scales—from milligram-level research batches to Good Manufacturing Practice (GMP) or commercial-scale production in the gram to kilogram range [35]. Scale-up introduces new engineering challenges such as oxygen transfer limitations, mixing heterogeneity, and shear stress gradients, which can influence plasmid topology and recovery yields. To ensure equivalence, manufacturers are expected to conduct comparability studies under the framework of ICH Q5E, evaluating physicochemical and functional attributes before and after process modifications [36]. Additionally, risk-based process validation—supported by statistical process control (SPC) and design of experiments (DoE)—is employed to confirm reproducibility of critical operations including fermentation, lysis, clarification, and purification [44]. Implementation of process analytical technology (PAT) further enhances real-time control and facilitates deviation management, ensuring product consistency across global manufacturing sites.

- **Emerging Manufacturing Innovations**

Recent advances in bioprocess engineering are reshaping plasmid DNA manufacturing, emphasizing efficiency, flexibility, and data-driven control. Continuous Bioprocessing is the integration of continuous fermentation and purification modules allows for steady-state operation, higher volumetric productivity, and smaller facility footprints compared to traditional batch systems [37,45]. Single-Use Technologies is an adoption of disposable bioreactors, filters, and membrane chromatography units minimizes the risk of cross-contamination and eliminates the need for extensive cleaning validation, accelerating changeover between campaigns [38,46]. Cell-Free DNA Synthesis Platforms are novel systems generate plasmid or linear DNA directly through enzymatic amplification or rolling-circle replication, circumventing bacterial hosts and thereby eliminating endotoxin and residual host DNA concerns [39,47]. Digital Twins and AI-Driven Monitoring are advanced computational tools now enable predictive control of CQAs, using digital twins and machine learning models trained on real-time sensor data to forecast supercoiled content, impurity trends, and process deviations with high accuracy [40,48]. Together, these innovations are driving the next generation of plasmid DNA manufacturing, aligning with the broader shift toward modular, automated, and data-

integrated bioprocessing platforms for advanced therapeutics.

## Regulatory Considerations

### FDA Perspective

In the United States, plasmid DNA (pDNA) used for advanced therapy medicinal products (ATMPs)—including viral vector gene therapies, mRNA vaccines, and DNA vaccines—is regulated by the Center for Biologics Evaluation and Research (CBER) of the U.S. Food and Drug Administration (FDA) under the biologics framework. The FDA classifies pDNA as a critical starting material, requiring full control and characterization consistent with Good Manufacturing Practice (GMP) expectations [21].

Key regulatory requirements include:

- **Supercoiled content and identity confirmation:** Manufacturers must demonstrate that the supercoiled (SC) isoform constitutes the predominant plasmid form (>80%), verified through agarose gel electrophoresis, capillary electrophoresis, or HPLC. This ensures the plasmid's functional integrity and transfection efficiency.
- **Residual impurity and endotoxin control:** Plasmid preparations must be extensively purified to remove host-cell DNA, RNA, and proteins, as well as endotoxins, which may trigger inflammatory responses. The FDA recommends orthogonal purification strategies and validated analytical assays for impurity quantification [22].
- **Sequence validation and stability:** The complete nucleotide sequence of each plasmid must be confirmed by Sanger sequencing or next-generation sequencing (NGS), and stability data must support the proposed shelf-life under defined storage conditions. Stability-indicating methods (e.g., restriction digest, potency assay) are essential for demonstrating product consistency throughout the lifecycle.

The FDA's Guidance for Industry: Considerations for Plasmid DNA Vaccines for Infectious Disease Indications (2007) and Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy IND Applications (2020) form the primary regulatory basis for pDNA submissions [21,22]. These emphasize a risk-based approach, encouraging early engagement with CBER for scientific advice and comparability discussions during technology transfer or scale-up.

### EMA Guidance

In the European Union, plasmid DNA used in ATMP production is governed by the European Medicines Agency (EMA) under the Advanced Therapy Medicinal Products Regulation (EC) No.

1394/2007 and related guidelines by the Committee for Advanced Therapies (CAT). The EMA classifies pDNA as a critical raw material, requiring comprehensive documentation and traceability from the source microorganism to the final product [23].

EMA guidance focuses on several specific regulatory expectations:

- **Documentation of cell banks and host strains:** Complete characterization of *Escherichia coli* host strains and master cell banks (MCB) must be provided, including details on genotype, antibiotic resistance markers, plasmid maintenance mechanisms, and absence of prophages or adventitious agents.
- **Antibiotic-free selection systems:** To minimize the risk of horizontal gene transfer and antibiotic resistance, the EMA increasingly favors selection markers such as auxotrophy complementation or operator-repressor titration, in place of antibiotic-based systems [23].
- **Comparability and scalability:** During process transfer or scale-up, comparability studies are required to demonstrate equivalence in plasmid yield, isoform composition, and impurity profile. The EMA expects these assessments to be supported by validated analytical testing, in line with ICH Q5E principles.

Furthermore, EMA's Reflection Paper on Quality, Non-Clinical and Clinical Aspects of Medicinal Products Containing Genetically Modified Cells (2018) extends the regulatory framework to pDNA-based intermediates, emphasizing batch-to-batch consistency, GMP compliance, and robust lifecycle documentation.

### ICH Harmonization

Globally, regulatory expectations for plasmid DNA manufacturing are guided by several International Council for Harmonisation (ICH) guidelines, which together support lifecycle management and cross-jurisdictional consistency:

- **ICH Q5A(R2) — Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin —** establishes principles for adventitious agent control, indirectly relevant to plasmid DNA used in viral vector production [7].
- **ICH Q6B — Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products —** defines analytical and quality standards for biological macromolecules, including criteria for identity, purity, potency, and stability applicable to plasmid-based materials [8].
- **ICH Q12 — Technical and Regulatory Considerations for Pharmaceutical Product Lifecycle Management —** enables structured man-

agement of post-approval changes via tools such as Post-Approval Change Management Protocols (PACMPs). This harmonization facilitates global adoption of manufacturing improvements while maintaining product quality [7,8].

Collectively, these ICH guidelines promote a science- and risk-based framework for ensuring the quality, safety, and consistency of plasmid DNA as a foundation for advanced therapeutics.

Despite major advances in purification and quality control, several technical and regulatory challenges persist:

- **Impurity Removal:** Achieving regulatory thresholds for residual RNA, genomic DNA, host-cell proteins, and endotoxins remains a bottleneck, especially at large scales [24].
- **Topology Control:** Maintaining high levels of supercoiled plasmid during processing is critical for potency but sensitive to shear, temperature, and pH variations [25].
- **Scalability:** Transitioning from research-scale (mg) to GMP-scale (g–kg) manufacturing often introduces yield variability and increases the complexity of process validation [26].
- **Regulatory Documentation:** Lifecycle management, comparability assessments, and GMP validation activities require extensive documentation, audits, and multidisciplinary coordination, adding significant time and cost burdens [27].

### Emerging Trends and Future Directions

The future of plasmid DNA manufacturing is being shaped by a convergence of technological and regulatory innovations:

- **Continuous Manufacturing:** Emerging membrane bioreactor and perfusion-based plasmid production platforms are improving productivity and reducing batch variability [28].
- **Cell-Free DNA Synthesis:** Enzymatic or synthetic biology-based systems are enabling antibiotic-free, endotoxin-free plasmid production, eliminating the reliance on *E. coli* hosts [29].
- **Digital Twins and AI Models:** The use of digital twins, combined with AI-driven predictive analytics, is revolutionizing real-time monitoring of critical quality attributes (CQAs) and critical process parameters (CPPs), enabling proactive quality control [30].
- **Regulatory Convergence:** Adoption of ICH Q12-driven lifecycle management frameworks, including PACMPs, is fostering global harmonization of plasmid DNA specifications and accelerating approval timelines for process improvements [31].

These trends collectively mark a paradigm shift toward data-integrated, flexible, and harmonized regulatory pathways, laying the foundation for the next generation of high-quality, scalable plasmid DNA manufacturing systems.

### Conclusion

Plasmid DNA has emerged as a pivotal raw material underpinning the manufacture of next-generation therapeutics—including gene therapies, mRNA vaccines, and genome-editing platforms. The transition from research-scale production to large-scale, GMP-compliant manufacturing demands a meticulous balance between bioprocess efficiency, product integrity, and regulatory compliance. Advances in host strain engineering, high-density fermentation, and multi-modal chromatography have significantly enhanced yield and purity, enabling the production of plasmids with >80–95% supercoiled content that meet global regulatory expectations. Nonetheless, persistent challenges such as impurity clearance, isoform control, and process scalability continue to define the developmental landscape of pDNA manufacturing.

The growing implementation of continuous and single-use bioprocessing, coupled with cell-free DNA synthesis platforms, offers a transformative path toward cost-effective, contamination-free, and sustainable production systems. Moreover, the integration of digital twins, machine learning, and process analytical technologies (PAT) is revolutionizing real-time monitoring and predictive control of critical quality attributes (CQAs) and critical process parameters (CPPs). These technological evolutions, aligned with harmonized regulatory frameworks—particularly ICH Q5A(R2), Q6B, and Q12—are fostering a global environment conducive to accelerated development, comparability, and lifecycle management of plasmid DNA-based therapeutics.

Looking forward, the convergence of data-driven automation, regulatory harmonization, and synthetic biology-driven process innovation is expected to redefine the plasmid DNA manufacturing paradigm. This evolution will not only ensure enhanced product quality, safety, and reproducibility, but also support the scalability and accessibility of advanced therapies worldwide—solidifying plasmid DNA's role as an indispensable enabler in the biomanufacturing ecosystem of the future.

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