Center for Breakthrough Medicines

Technical White Paper

Manufacture the Highest Quality Precision Plasmids™ with a Reliable Platform Process

Outline

Author:
John Bowen, Sr. Director, Nucleic Acids and Plasmid Operations
Avi Nandi, Chief Technical Officer
Dana Cipriano, Sr. Vice President, TAS & Nucleic Acids
Joseph Dang, Sr. Director, Product Management

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THE GROWING DEMAND FOR PLASMIDS

Since the first pharmaceutical application of plasmids (their introduction into bacteria to enable the production of therapeutic proteins) was achieved in the 1970s, **plasmids have become critical starting materials for many advanced drug products**. They are used to transfect mammalian cells to manufacture complex recombinant proteins, antibodies, and other biologic drug substances. With the increasing investment in advanced therapies such as viral vectors, cell-mediated, and nucleic acid-based modalities, plasmid manufacturing has become a major bottleneck to deliver these therapies. Plasmid transfection into mammalian cells is the predominant production method for viral vectors. Plasmids also serve as the template for mRNA manufacturing and have potential applications as vaccines and other therapeutics.

Current gene therapies treat rare diseases with small patient populations (<10,000).[1] Small-scale production platforms are sufficient to meet plasmid needs for these products. Approximately 60% of gene and gene-modified therapy candidates in the pipeline, however, address more prevalent indications.[2] It is estimated that viral vector marketed products could require 100–1,000 g of plasmid per year.[1] In addition, delivering one billion doses of an mRNA vaccine, meanwhile, can consume more than 1 kg of plasmid DNA.[3]

Given the wide range of applications for these double-stranded, circular pieces of DNA, plasmids have become essential building blocks for the manufacturing of advanced therapies. **A significant percentage (between 80 to 90%) of plasmids used in the biopharmaceutical industry are produced by contract development and manufacturing organizations (CDMOs)** with the necessary specialized expertise.[1] Consequently, there has been a strong rise in demand for plasmid production services. The value of the global plasmid DNA manufacturing market is expected to expand at a compound annual growth rate of nearly 14% to reach $1.51 billion by 2030.[4]

The rapid increase in demand is causing plasmid supply and manufacturing to become a critical pain point in the industry. As the supply of plasmids has become more crucial, complex supply-chain issues that arose during the COVID-19 pandemic continue to linger. **Wait times for access to good manufacturing practice (GMP) plasmid production capacity often exceed 12 months**[5], which can lead to delays in the progress of late-stage clinical (phase III) programs and those nearing commercialization. In addition, it is increasingly common for drug developers to use GMP-grade plasmids for phase I/II trials on expectations of forthcoming regulatory requirements, further increasing demand.[6]

Recognizing the need for additional plasmid manufacturing capacity, CBM currently has 2,000 SF of GMP capacity with up to a 50L fermentation scale. Construction is also underway for a 20,000+ square-foot GMP facility with ISO classified cleanrooms built for plasmid and mRNA production.

PLASMID MANUFACTURING CHALLENGES

To meet the rising demand for plasmid DNA, process scaleup and optimization of current production processes is needed as both upstream and downstream challenges exist. Developers should put attention to four critical areas:

1. Lysis

Upon successful *E. coli* strain selection and fermentation process development, significant challenges revolve around *E. coli* lysis. Lysate can be very dense and highly viscous, making it-difficult to harvest and clarify. In addition, these unit operations are time-dependent and plasmid degradation can occur if routine processing times are exceeded.

2. Negative charge

Plasmids are also highly negatively charged over a broad pH range and are sensitive to degradation by nucleases. In clarified lysates, desired plasmid products typically make up <3% of the material, and many of the impurities (e.g. RNA, genomic DNA, host-cell proteins, endotoxin, unwanted plasmid isoforms: open circular and linear vs. supercoiled) generated during fermentation need to be removed via a downstream purification process. Yields of purified product are therefore low (typically <1% dry cell mass).[7]

3. Shear sensitivity

The shear sensitivity of plasmids must be taken into account during all operations in order to minimize degradation and maximize recovery. In combination with the high viscosity of *E. coli* lysate, plasmids are susceptible to degradation dependent on plasmid size, buffer ionic strength, and liquid/air interface interactions during processing. The large size of plasmid DNA (typically 5–kilobase pairs, but trending towards larger constructs[7]) poses difficulties for downstream purification.

4. Regulatory guidelines

FDA guidance for DNA vaccines[8] (established in 2007) are typically followed for plasmid DNA as well, requiring a minimum 80% supercoiled DNA. Expectations have increased, however, and there is a **general understanding in the industry today that the percentage of supercoiled isoforms should be >90%**.[1]

Overall, plasmid DNA production processes are somewhat expensive and lengthy, including long wait times, *E. coli* strain selection, process development, analytical qualification, and subsequent manufacturing and release testing. There is significant need to increase scalability, efficiency, and productivity.

To provide a solution to these challenges, CBM has developed a platform manufacturing process for a variety of plasmid sizes and sequence complexities with platform analytical release methods.

CBM'S PLASMID MANUFACTURING PROCESS

The plasmid DNA manufacturing process **(Figure 1)** employed at CBM can be performed in a closed single-use fermenter (50L – 200L). Media and nutrients are sterile-filtered or thermally sterilized and aseptically transferred into the fermenter, which is operated in batch and fed-batch mode to optimize cell mass generation and plasmid production, respectively. Post fermentation, a batch centrifuge or disposable continuous centrifuge is used (depending on scale) to sediment and collect the *E. coli* cells. The cell paste is then resuspended using an optimal buffer and dilution ratio where the intracellular plasmid is released via chemical cell lysis. Lysate is then clarified to remove cell debris and larger impurities via depth filtration.

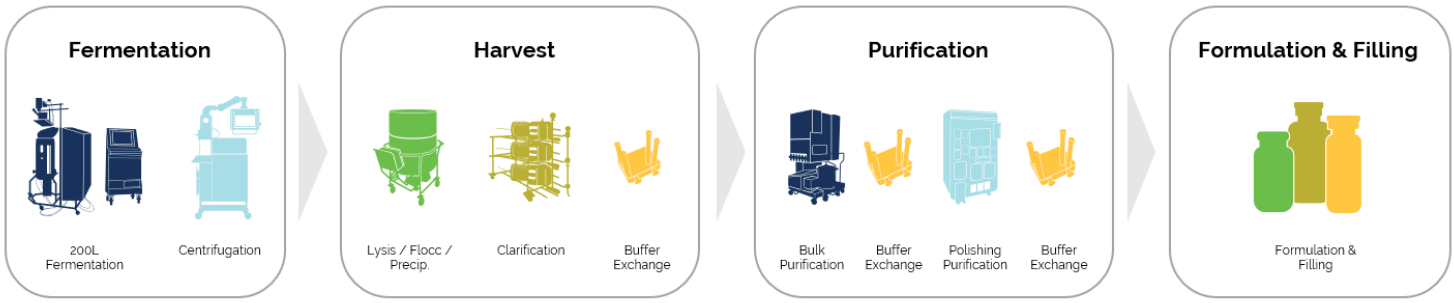


Figure 1: Plasmid production process

Downstream purification involves multiple tangential flow filtration (TFF) and chromatography steps (anion exchange/AEX for bulk purification and hydrophobic interaction/HIC for polishing) to ensure product quality. TFF is required to concentrate the harvest fluid and perform a buffer exchange prior to anion exchange chromatography. It is also used after the polishing chromatography step to perform the final buffer exchange. For chromatographic purification, rather than use traditional bead-based resins, monolith chromatography is employed. These monolith columns are available in a variety of pore sizes, allowing proper sizing to achieve optimal dynamic binding capacities for each individual plasmid. Finally, a sterile filtration is then performed prior to filling into a variety of containers (bags, bottles, or vials).

> **CBM's platform is designed to deliver high quality GMP plasmids in 6 weeks from a starting master cell bank or 18 weeks from a starting R&D plasmid**

Building Critical Process Parameter Know-How

The goal at CBM is to establish a platform pDNA production process that is robust, scalable, and generates high quality pDNA that meet stringent regulatory requirements. To develop such a manufacturing platform requires in-depth knowledge of the upstream, mid-stream, and downstream unit operations (specifically when different *E. coli* strains and a range of plasmids are used).

A design-of-experiment (DoE) approach was taken to characterize processes and determine critical process parameters (CPPs) that impact critical quality attributes (CQAs) for *E. coli* fermentation and downstream plasmid purification. Subsequent data will highlight critical process parameters and their impact on both yield and quality.

To develop a flexible platform, the upstream DoE approach explored different plasmid characteristics (size, complexity, and origin of replication) and multiple *E. coli* production strains. Four different plasmid sequences were investigated. Three plasmids representing commonly used sequences for the production of recombinant AAV vectors, including pRepCap, pHelper, and pGOI (gene of interest) were chosen. The pGOI construct represents the complex secondary inverted terminal repeat (ITR) sequence that can be difficult to properly amplify due to its complex repeating structure. The fourth plasmid was a green fluorescent protein (GFP) reporter plasmid with long terminal repeat (LTR) sequences for lentiviral (LV) vector production.

> **Upstream DOE** was conducted on plasmid size, complexity, & origin of replicaiton along with DH5α and Stbl™ *E.Coli* strains

Several *E. coli* strains were used to produce the four plasmids. DH5α, for which CBM has a license, was of primary interest. It provides higher yields but can be prone to amplification errors when replicating DNA sequences with unstable inserts such as direct repeats or high GC content regions. Stbl strains were also evaluated as they are known to result in high fidelity plasmid amplification leading to higher quality. *E. coli* strain screening was executed for each plasmid prior to fermentation optimization.

Upstream process parameter optimization included media formulation, carbon source selection, fed-batch feeding profile (linear, constant, exponential), temperature, pH, dissolved oxygen and the use of a heat shift.

> **Seven upstream CPPs -** media formulation, carbon source selection, fed-batch feeding profile, temperature, pH, dissolved oxygen, and heat shift usage - were optimized.

Optimization of the cell lysis step is also crucial for achieving maximum productivity and maintaining high quality supercoiled plasmid topology. CBM has optimized critical process parameters, and has also demonstrated the ability to continuously process *E. coli* lysate leading to high quality and reproducible results during scale-up.

After optimizing the upstream and mid-stream unit operations, the purification process was optimized using a two-column chromatography process. Anion exchange chromatography was used to capture pDNA while removing the majority of RNA contaminants. Subsequently, a hydrophobic interaction chromatography step was developed to enrich supercoiled plasmid topology to >95%. This development approach has led to a platform process that will enable high quality, robust, routine manufacturing.

Optimizing Fermentation

In-line critical process parameters (agitation, pH, dissolved oxygen, & turbidity) are monitored during the fermentation. In addition to optimizing fermenter operating parameters, multiple other conditions such as the base media selection, carbon source, feeding strategy, and the batch to fed-batch transition were investigated. A sub-set of data is presented in **Figure 2** below.

Medium	Carbon Source concentration [g/L]	Time since feeding start	OD600	Vol. Plasmid Yield [mg pDNA/L]
Medium 1	2X	Late	3.20X	7.11X
Medium 2	1X	Late	2.24X	3.96X
Medium 3	2X	Early	1.12X	1.58X
Medium 4	2X	Early	1.00X	1.00X

Figure 2: Plasmid fermentation optimization using carbon source concentration and feeding strategy as an example

Of the four media investigated, Medium 1 and 2 provided the best volumetric productivity (mg pDNA/L) indicating that greater carbon-source concentrations and a longer "batch phase" to increase cell mass prior to plasmid production were important factors. Notably, a 7.11X increase in productivity was observed from the least productive to most productive condition. This highlights the importance of fermentation optimization in plasmid manufacturing.

In a separate set of experiments, multiple feeding profiles during the fed-batch portion of the fermentation were investigated. Since *E. coli* grow exponentially, feeding media at an exponential rate to keep up with their metabolic demand ensures that media components are added at a similar rate relative to the number of cells present. However, simpler feed strategies such as a linear increase or constant feed were also investigated. **Figure 3** summarizes a sub-set of the feeding strategy optimization study.

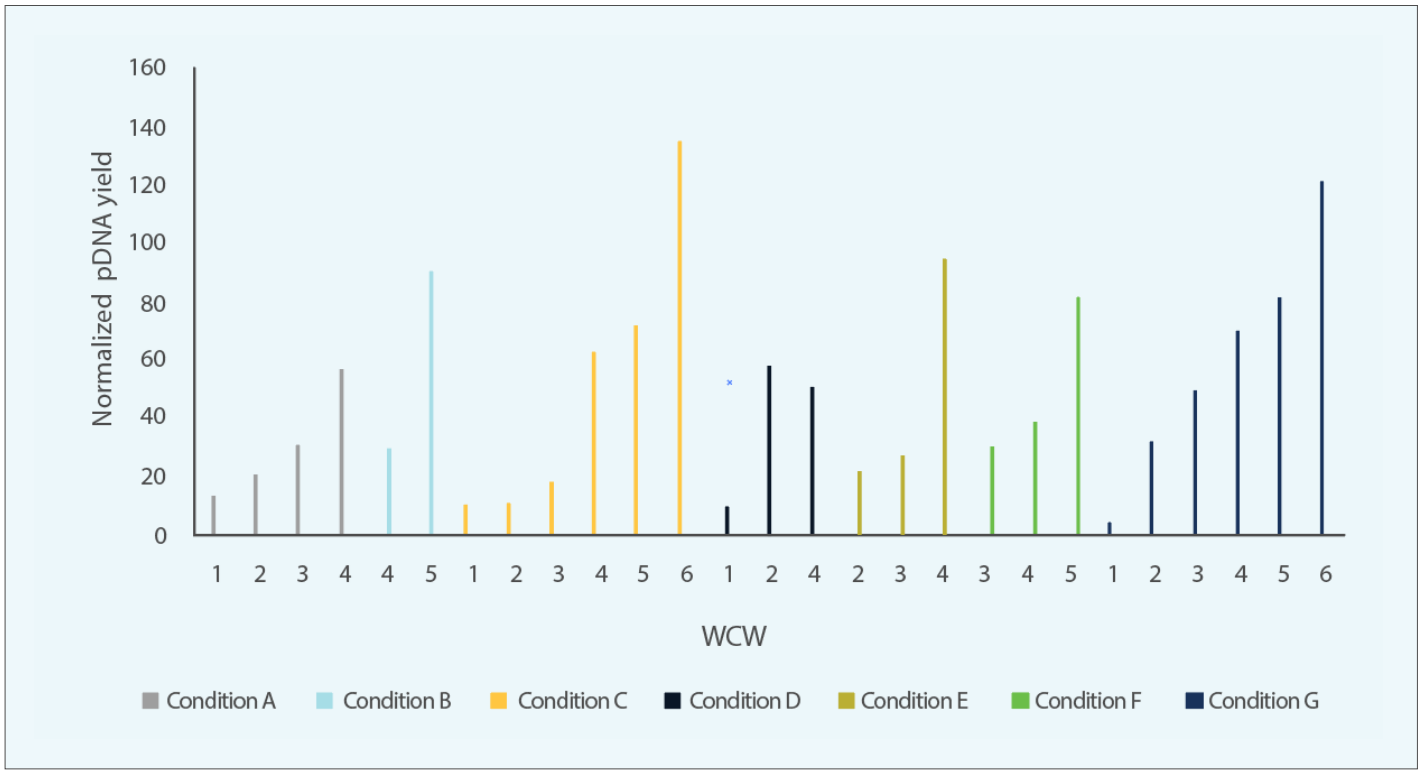


Figure 3: Plasmid productivity for seven different feeding profiles

A sub-set of the results are shown for 4 exponential feeds, 2 combination feeds, and 1 linear feed in **Figure 3**. The normalized final cell mass in weight cell weight (WCW) is shown on the x-axis. Not surprisingly, longer fermentation durations and higher weight cell weights within a given condition yielded higher productivities. Also, note the large differences in final WCW for different feeding conditions. **Finally, while comparing the productivities at the greatest WCW, there are stark differences in productivity (greater than 2X increase from the best condition to the worst condition).**

Next, the impact of the feeding strategy in conjunction with the fermentation temperature was investigated. A feed strategy was chosen as the baseline and four temperatures (low, medium-low, medium-high, and high) were investigated. **Figure 4** summarizes the productivity data for different temperature conditions. Similarly to **Figure 3**, the highest WCW led to the highest productivity within a given condition. Also, the "medium-high" temperature in Condition C yielded the best productivity whereas the "low" temperature in Condition A performed the worst.

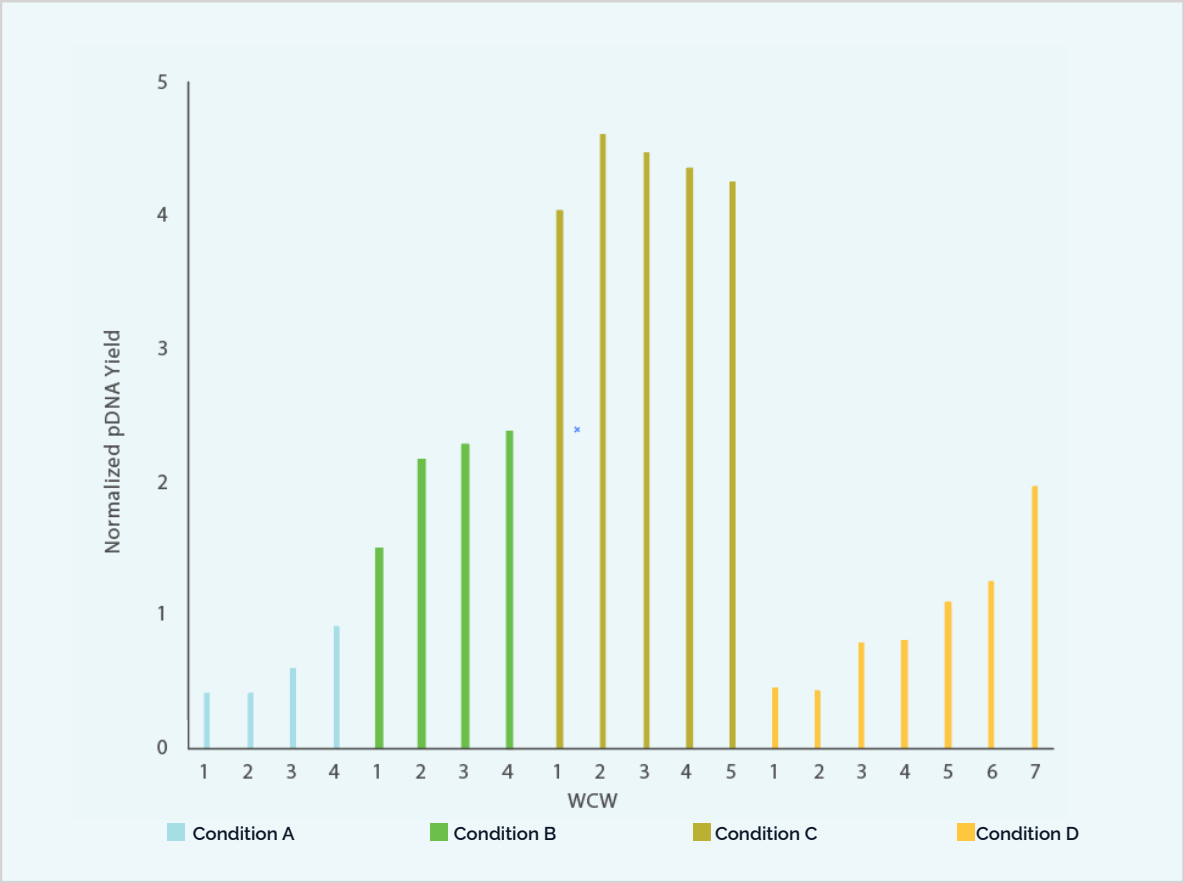


Figure 4: Comparison of four temperature conditions during fermentation

This data highlights the importance of media selection, fed-batch strategic implementation, and temperature optimization. These parameters, in addition to multiple other critical processing parameters (data not shown) have led to a platform fermentation process. Similar data sets for other plasmids and *E. coli* strains have been generated to ensure a robust data set exists for future manufacturing needs.

Optimizing Harvest
Lysis, neutralization, and flocculation – Batch

E. coli lysis following fermentation is necessary to liberate the plasmid from *E. coli*. The cells are separated from the rest of the fermentation broth via centrifugation. The cell paste is then resuspended in an appropriate buffer. The pH, incubation time, and mixing must be carefully controlled to prevent irreversible denaturing and/or degradation of the plasmid to unwanted topologies such as open circular or linear DNA. The lysis mixture is then neutralized using a solution to cause agglomeration and precipitation of unwanted impurities. The aggregates eventually precipitate while the circular plasmid DNA remains in solution.

During the lysis unit operation, the cell paste resuspension volume and the lysis duration were analyzed via a 2-dimensional DoE approach. Three cell resuspension ratios and three lysis durations (Low, Medium, High) were investigated (**Figure 5**). Higher resuspension ratios resulted in increased yields and higher plasmid quality. This conclusion was observed regardless of the lysis time. Regarding the lysis time, a similar phenomenon was observed where the longest lysis time led to the greatest yield and highest plasmid purity. After this observation, other studies were conducted to increase the lysis time beyond this "high" value until yields decreased and lower plasmid quality was observed.

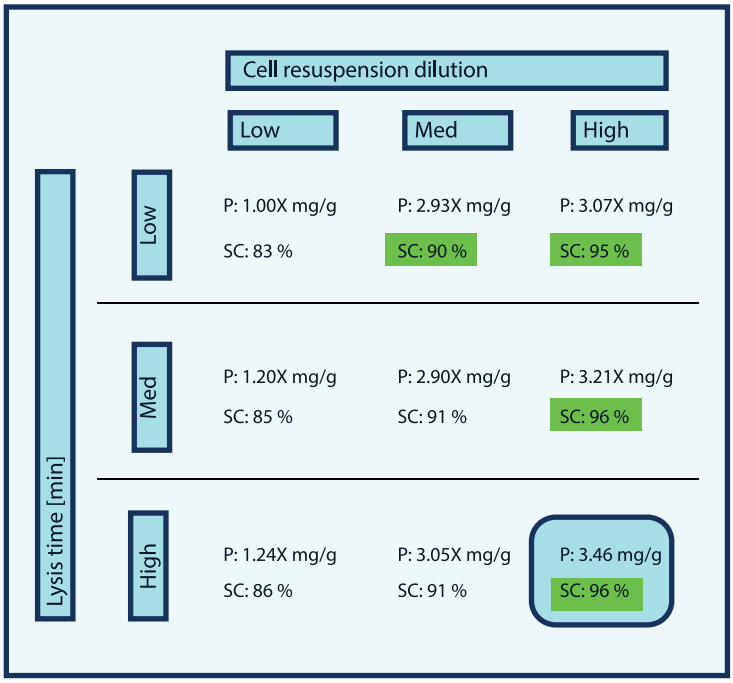
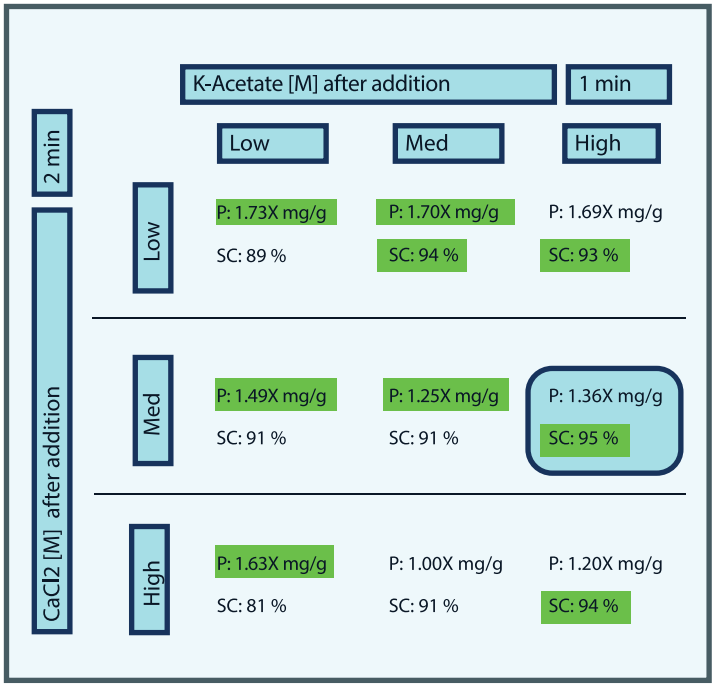


Figure 5: 2-Dimensional DoE approach for optimization of the batch-mode lysis step. The productivity (mg plasmid / g cell paste) and the purity (% supercoiled) are presented

Post lysis, subsequent neutralization and flocculation steps are performed to aggregate impurities released from the *E. coli* cells. A similar 2 dimensional DoE approach was conducted to determine the optimum conditions for the neutralization/flocculation step (**Figure 6**). In this study, three different concentrations (Low, Medium, High) were investigated.

The highest K-Acetate concentration provided efficient and rapid neutralization combined with higher plasmid quality, while lower concentrations led to a reduced percentage of supercoiled pDNA while seeing a minimal impact on yield. The prolonged exposure to alkaline conditions could lead to plasmid degradation explaining lower amounts of desired supercoiled plasmid. Regarding the subsequent flocculation step, the highest concentration resulted in fewer RNA impurities (data not shown) but led to a greater amount of pDNA precipitation causing lower yields. The "Med" condition was chosen to maintain the highest quality while ensuring minimal yield impact.

Figure 6: 2-Dimensional DoE approach for optimization of the Neutralization/Flocculation unit operation. The productivity (mg plasmid/g cell paste) and the purity (% supercoiled) are presented



Continuous lysis/neutralization/flocculation

Minimizing the time that plasmid DNA is exposed to harsh conditions during lysis is essential for preventing its degradation. Achieving this goal when performing these operations in **batch mode can be challenging** at large scale due to the significant volumes that must be manipulated and the short durations that need to be achieved.

A continuous lysis/neutralization/flocculation solution can be implemented to circumvent these scale-up or scale-out manufacturing issues. Similar to the individual manipualations of the batch process, the overall continuous process was optimized using a DoE approach.

After optimization of the process, it was determined that in-line lysis/neutralization/flocculation is comparable to the batch approach (**Figure 7**). The plasmid DNA yield was slightly higher (1.2X vs 1.X and the quality was higher (98% vs. 95% supercoiled). Other impurities such as multimer DNA and residual RNA also showed a slight improvement. **Continuous processing ensures easier scalability and provides more control than traditional batch processing**.

Figure 7: Comparison of optimized batch and continuous lysis/neutralization/flocculation performance

	Yield	Supercoiled (%)	Multimer	Residual RNA
Batch	1X	95	1X	1X
In-line	1.2X	98	0.9X	0.85X

Clarification

Clarification is performed to remove cell debris and other impurity aggregates generated during the lysis step. This is achieved via depth filtration, and multiple filter pore sizes, surface areas, and vendors were screened during this unit operation development (data not shown). A filter was chosen with a pDNA recovery of 90% while maintaining 93% supercoiled plasmid topology in the clarified material.

Buffer Exchange and Concentration Prior to Capture Chromatography

After the pDNA lysate is clarified, a concentration and buffer exchange step is required prior to the anion exchange capture chromatography operation. During initial stages of small-scale development, the clarified lysate was diluted to a specific conductivity prior to loading onto the anion exchange chromatography resin. However, during scale-up, it was immediately determined that a buffer exchange and concentration step would lead to more scalable operations.

A tangential-flow filtration (TFF) step was therefore introduced to reduce the volume and exchange the buffer solution to achieve the proper conductivity. In addition to providing a more manageable volume for downstream purification, this step affords greater process control by ensuring a consistent feed into the capture chromatography step.

Optimizing Downstream Purification

In the CBM pDNA platform manufacturing process, downstream purification operations include bulk purification via Anion Exchange Chromatography (AEX) to remove RNA and other process-related impurities followed by polishing purification via Hydrophobic-Interaction Chromatography (HIC) to remove unwanted plasmid isoforms such as open circular plasmid. The polished material is then buffer exchanged into the desired buffer and sterile filtered into a variety of container options.

Bulk Purification

AEX is performed as the bulk capture chromatography operation. The loading condition and dynamic binding capacity was initially identified (data not shown). After optimization of the load, wash, and elution conditions, these fractions were processed via HPLC to determine the plasmid isoform distribution. Data in **Figure 8** indicate the yield and quality profile for three separate AEX runs. Notably, the desired supercoiled topology is greater than 90% for each. This high quality plasmid exceeds supercoiled acceptance criteria after the first chromatography step.

Figure 8: Anion exchange chromatography performance comparison

Yield (%)	Supercoiled (%)	Open Circular (%)	Linear (%)
96	91	9	0
94	96	2	2
96	93	5	2

Polishing Purification

After AEX chromatography, the collected fraction is further purified using hydrophobic interaction chromatography. This polishing step is necessary to remove endotoxin, genomic DNA, and undesired pDNA isoform impurities. Similar to AEX, after optimization of the load, wash, and elution conditions, these fractions were processed via HPLC to determine the plasmid isoform distribution. As the final polishing step, specific attention to plasmid quality is important. Step yields and product quality are shown in **Figure 9**. The supercoiled pDNA topology is enriched to greater than 95% for each run.

Yield (%)	Supercoiled (%)	Open Circular (%)	Linear(%)
94	99	1	0
93	99	1	0
97	95	4	1

Figure 9: Hydrophobic interaction chromatography performance comparison

PLATFORM PERFORMANCE RESULTS

Results from CBM's platform downstream purification process is summarized below. Productivity, plasmid quality, and multiple residual impurities throughout the process are shown.

Unit Operation	Productivity per Cell Weight [mg/g]	Yield [%]	Amount Supercoiled [%]	Endotoxin Level [EU/mg pDNA]	gDNA/pDNA [%]	Proteins/pDNA [%]
Lysis	2.63	N/A	95	2089	1.3	N/A
Clarification	N/A	90	93	291	0.2	2.74
TFF		95	97	Not Recorded		
Capture Chromatography		96	93	27	0.2	~0.01
Polishing Chromatography		95	99+	1.25	0.03	~0.01

The extensive DoE studies elucidate optimum process parameters for production of different plasmid types and have revealed several important concepts that must be taken into consideration when implementing a platform manufacturing solution. This results in over 95% supercoiled topology (compared to 80% industry) and endotoxin levels 32X lower than FDA regulatory guidance. In addition, plasmid identity is guaranteed via next generation sequencing.

Most importantly, not all plasmids have comparable fermentation yields, with yields ranging from 0.2 – 1.5 g/L depending on plasmid sequence, *E. coli* production strain selection, and fermentation processing parameters. While it is possible to develop a platform process, operating parameters within the overall process must be sufficiently flexible to account for the variety of plasmid sizes and sequences that will be observed. CBM's approach to developing a platform process has provided a valuable dataset to de-risk any future plasmid manufacturing needs.

Clients of CBM seeking plasmid manufacturing services will benefit from this optimal, robust, efficient, and scalable platform for their pDNA needs. Our R&D and manufacturing experts are well-versed in the challenges that must be addressed when developing large-scale plasmid production processes and work closely with customers to rapidly identify effective solutions to any issues that arise.

REFERENCES

1. Tony Hitchcock, "Manufacturing Plasmid DNA: Ensuring Adequate Supplies for Gene and Cell Therapies," BioProcess International, October 17, 2016. https://bioprocessintl.com/manufacturing/cell-therapies/manufacturing-plasmid-dna-ensuring-adequate-supplies-gene-cell-therapies/

2. Jonny Ohlson, "Plasmid manufacture is the bottleneck of the genetic medicine revolution," Drug Discov Today. 2020 Nov; 25(11): 1891–1893. doi: 10.1016/j.drudis.2020.09.040

3. Grand View Research, "Plasmid DNA Manufacturing Market Size Worth $1.51 Billion by 2030," Press Release, October 5, 2022. https://www.prnewswire.com/news-releases/plasmid-dna-manufacturing-market-size-worth-1-51-billion-by-2030-grand-view-research-inc-301640683.html

4. Mordor Intelligence, "Viral Vector & Plasmid DNorth America Manufacturing Industry Overview" https://www.mordorintelligence.com/industry-reports/viral-vectors-and-plasmid-dna-manufacturing-market

5. Alejandro Becerra and Johannes F Buyel, "Optimizing downstream purification of high-quality plasmid DNA for gene therapy and vaccine production," Cell & Gene Therapy Insights 2021; 7(11), 1217–1230. DOI: 10.18609/cgti.2021.162

6. Lalintip Hocharoen, Sarawuth Noppiboon, and Panit Kitsubun, "Process Characterization by Definitive Screening Design Approach on DNA Vaccine Production," Front. Bioeng. Biotechnol., 15 October 2020. Sec. Bioprocess Engineering . https://doi.org/10.3389/fbioe.2020.574809

7. Aaron E.E. Carnes and James A. A. Williams, "Low Metabolic Burden Plasmid Production," Gen Eng. News. March 1, 2009. https://www.genengnews.com/magazine/108/low-metabolic-burden-plasmid-production/

8. Considerations for Plasmid DNA Vaccines for Infectious Disease Indications. November, 2007. https://www.fda.gov/files/vaccines,%20blood%20&%20biologics/published/Guidance-for-Industry--Considerations-for-Plasmid-DNA-Vaccines-for-Infectious-Disease-Indications.pdf

Center for Breakthrough Medicines

ABOUT THE CENTER FOR BREAKTHROUGH MEDICINES

CBM is a cell and gene therapy contract development and manufacturing organization (CDMO) based in the heart of Philadelphia's Cellicon Valley. CBM offers preclinical through commercial manufacturing capabilities including process development, plasmid DNA, viral vector manufacturing, cell banking, cell processing, and a full suite of complimentary testing and analytical capabilities. Through a single-source, end-to-end solution, CBM accelerates time to market without compromising quality.

Co-locating manufacturing, process development and analytical services prevents delays and handling errors. CBM's aim was to create one campus, one building, one manufacturing site. Our purpose built 700,000 sq. ft. manufacturing center is future-proofed in terms of infrastructure within and around the site. The current facility sits on over 1 million sq. ft. of space, allowing for future expansion to match the growing demand of the cell and gene therapy industry. Internally, the suites have been designed so that complementary services and labs are adjacent or nearby, to ensure we can accelerate time to market without compromising quality.

Scan or click the QR code to learn more about our plasmid DNA manufacturing

AUTHORS

John Bowen
Sr. Director, Nucleic Acids & Plasmid Operations

Avi Nandi
Chief Technical Officer

Dana Cipriano
Sr. Vice President, Nucleic Acids & TAS

Joseph Dang
Sr. Director, Product Management