# Purification Process Development of Plasmid DNA for Gene Therapy

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## Abstract

Plasmids are small double stranded DNA molecules that are separate from the chromosome. Plasmids are present naturally in bacterial cells and are commonly used as raw materials in mRNA and gene therapy vaccines. These plasmids can be used as the gene template for mRNA vaccines, or to provide the necessary genes, such as rep/cap/gene of interest, in adeno-associated virus (AAV) processes.

The current work outlines MilliporeSigma's collaboration with Boston Institute of Biotechnology, to develop unit operations for the purification of a plasmid DNA used for AAV gene therapy application. Currently the purification of pDNA in a pure form ready for therapeutic use still presents various challenges starting from the lysis step. It also requires development of highly reproducible and scalable processing methods that meet regulatory manufacturing standards. The development here focuses on purification of the supercoiled isoform of pDNA and the use of scalable technologies on process steps of lysate clarification, ultrafiltration/diafiltration, capture using membrane ion exchange chromatography, and sterile filtration.

## Introduction

**Plasmid DNA:**

- Short circular pieces of DNA found naturally in *E. coli*
- Able to replicate independent of the chromosome
- Easy to manipulate molecule for replication of genetic material
- Used in in/ex-vivo gene therapy, mRNA vaccine, and plasmid vaccine

**Boston Institute of Biotechnology LLC (BIB),** is a biotechnology contract development and manufacturing organization (CDMO). We offer full spectrum CMC services on AAV, microbial and mammalian production.

**pDNA and AAV Services:**

- Vector Design
- pDNA Production
- AAV Host Cell Screening
- AAV Process Development
- AAV GMP production BL2 suites
- 4×2,000L GMP Cell Culture Line
- 2×2,000L GMP Microbial Line

## Methods

**Clarification: Constant Flow Pmax™ method**
Clarification was evaluated using the constant flow Pmax™ method. Depth filters were flushed with 100 L/m$^2$ DI water, and 50L/m2 TE buffer at a flux of 600 LMH [L/m$^2$/hr, 23mL/min] prior to use.

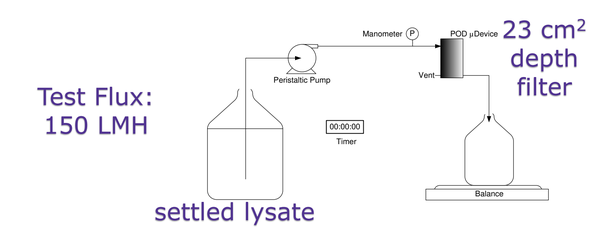
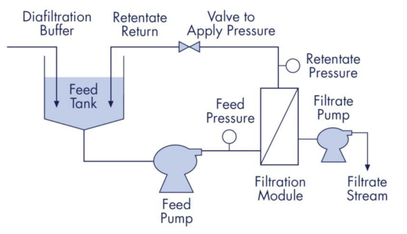



**Image 1: Schematic of a constant flow depth filtration experiment**

**Ultrafiltration/Diafiltration: 2-Pump TFF**
Ultrafiltration/diafiltration (UF/DF) was evaluated with a 2-pump system (see figure). Pellicon® XL50 cassettes with Biomax® 100kDa and 300kDa membrane were used. The device was cleaned with 0.2N NaOH before use.




5 L/min/m$^2$ cross flow
20 L/m$^2$/hr permeate flux
50 cm$^2$ filter
10x UF and 5x DF

**Chromatography:**

- Natrix® Q recon mini format devices were used (0.2 mL each)
- Target flow rate for each step was 2 mL/min or 10 membrane volume (MV)/min
- A linear gradient salt elution was run over 30 MV

Equilibrate 50 MV → Load 40mL, 10 g/L → Wash 20 MV → Elute 30 MV → CIP 30MV

**Table 1: Chromatography & UF/DF Buffers**

| Property | Value |
|---|---|
| **pDNA Size** | 22 kbp |
| **Settled Lysate Turbidity [NTU]** | 157 NTU |
| **Lysate Resuspension Concentration [g/L]** | 50 g/L |
| **Resuspension Buffer** | 50 mM Tris, 100 mM EDTA pH 8 |
| **Diafiltration Buffer/Chromatography Equilibration** | 1M Potassium Acetate + 147 mM NaCl pH 4.85, 76 mS/cm |
| **Natrix® Wash Buffer** | 100 mM Sodium Acetate, pH 5.0 |
| **Natrix® Elution Buffer** | 100 mM Sodium Acetate, 1M NaCl, pH 5.09 |

## Results

**Lysate Clarification:**

Pressure profiles for each depth filter are shown in Figure 1A. Figure 1A shows low pressure for all depth filter grades except filter CE25. Volumetric throughput for these conditions was >300 L/m$^2$. Most conditions showed similar filtrate DNA concentration to the lysate load sample. A significant reduction in lysate DNA concentration was observed following calcium chloride precipitation with 1.4M dosage (Figure 1B).

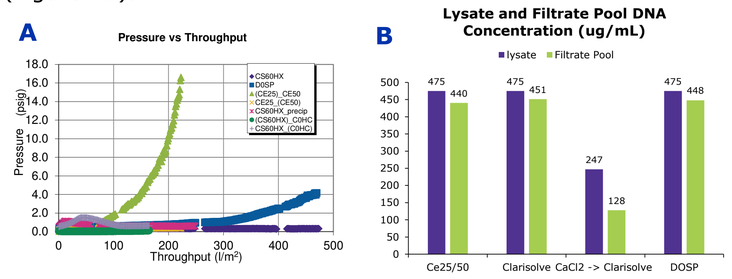



**Figure 1: Clarification Results**
Comparison of pressure vs throughput (A) & Pool DNA concentration (B)

**Calcium Chloride Precipitation:**

The target plasmid is around 22KB in size, and the RNA is shown in the bottom bands. Figure 2 indicates the RNA quantity is reduced with calcium chloride concentrations above 0.4M. At higher concentration, plasmid DNA is also reduced.

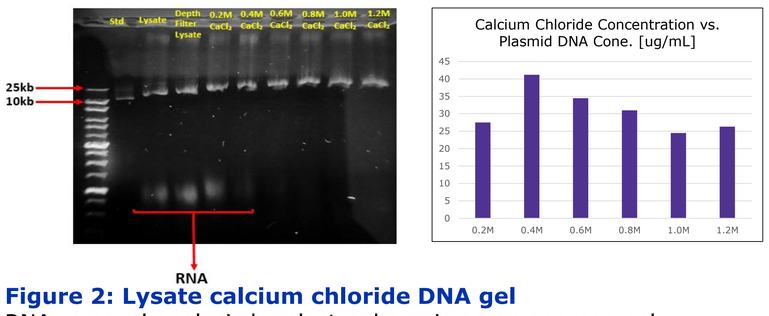



**Figure 2: Lysate calcium chloride DNA gel**
RNA removal analysis by electrophoresis on an agarose gel

**Ultrafiltration/Diafiltration with Biomax® 100/300kDa:**

Critical flux is 20 LMH for the 100kDa membrane. Critical flux was not exceeded for 300kDa membrane (Figure 2A). Permeate flux is reduced on 100kDa membrane at 3.5x concentration factor (Figure 2B). Improved pressure during diafiltration is observed for the 300kDa membrane (Figure 2C). Protein and DNA are both concentrated during the UF/DF experiments. DNA is not permeating as shown by gel analysis (Figures 2D,E,F). RNA appears to decrease after diafiltration, the higher salt condition might be facilitating this observation (Figure 2E).

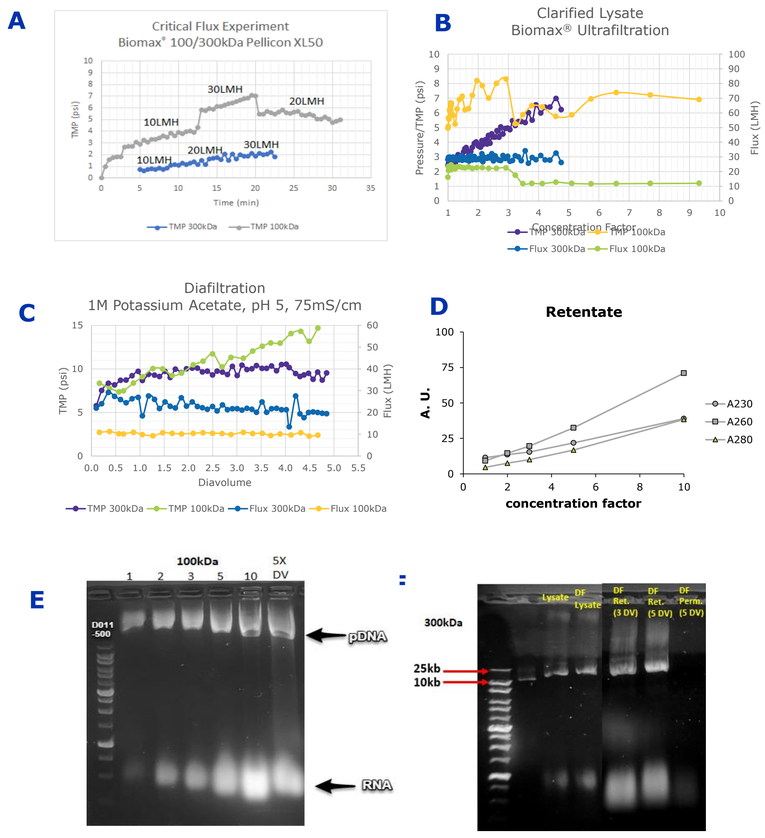
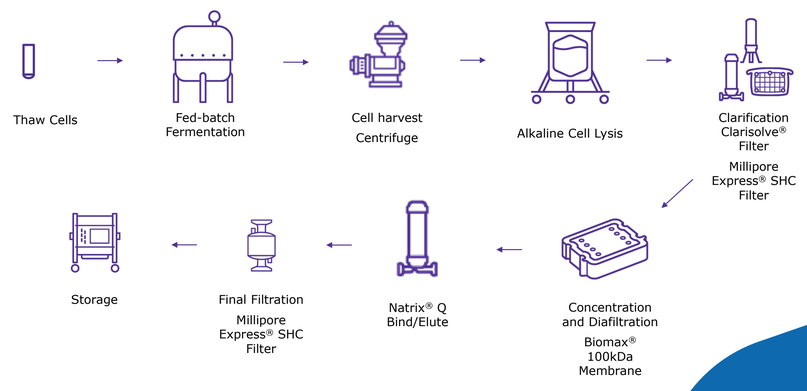


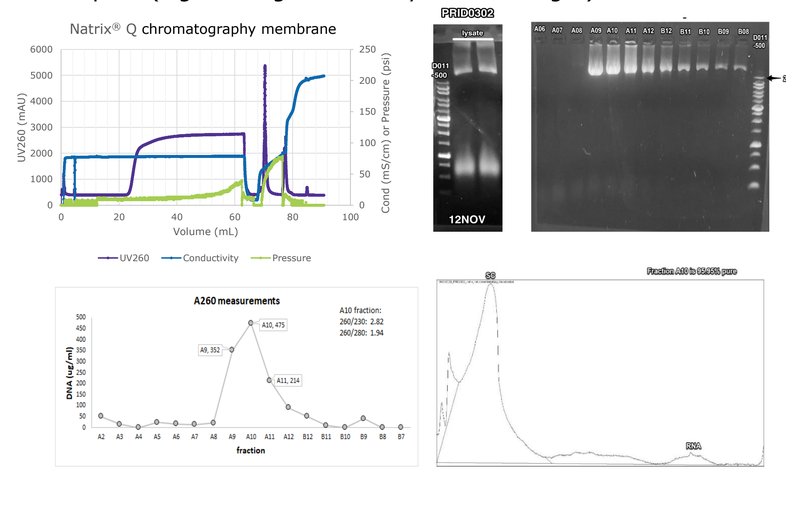

**Figure 2: Ultrafiltration Diafiltration Results**
Plot of filter pressure and flux during critical flux, ultrafiltration, and diafiltration (A-C), UV absorption for 100kDa retentate samples (D), gel analysis for samples (E), and UV absorption for permeate samples (F)

## Process Flow Path

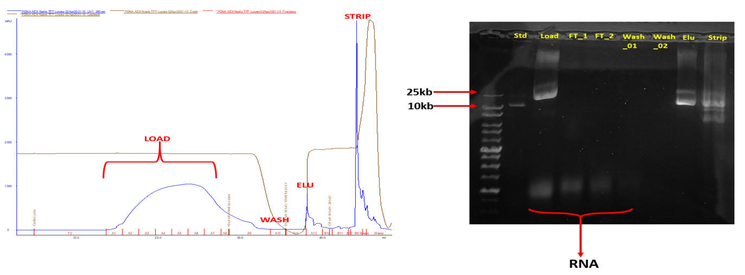



**Chromatography:**

**A Natrix® Q chromatography membrane (lysate)**
A sharp elution peak is observed based on UV260 signal (Figure 3A left). Pressure increase was observed during the load step and can be mitigated by reducing the load volume. Gel analysis indicates the plasmid is concentrated in 4 of the collected fractions, UV260 analysis are consistent with gels. Fraction A10 is 95% pure (Figure 3A gel and analysis on the right).




**B Natrix® Q chromatography membrane (TFF concentrated)**
Gel analysis for the flow through samples shows some RNA and no pDNA. The strip peak was larger than the elution peak indicating strong binding conditions. The elution peak shows an intense pDNA band along with potential presence of isoforms.




**C Fractogel® DMAE resin** results suggest breakthrough of pDNA occurred during the load step, as shown by UV260 profile. Fractogel® gel analysis indicates presence of pDNA isoforms such as open circular (Figure 3C). Sample E7 is 78% pure.

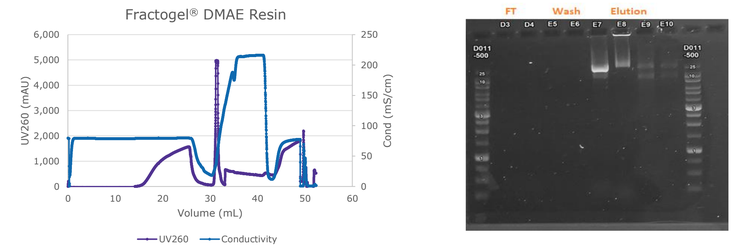



**Figure 3: Chromatography Bind/Elute Results**
Plot of UV, pressure, and conductivity for Natrix® Q native lysate experiment (A), Natrix® Q chromatography membrane with TFF concentrated sample (B), and Fractogel® DMAE resin with native lysate for load

## Summary

- Clarification: Filtration with Clarisolve® 60HX depth filter grade followed by Millipore Express® SHC PES membrane provides high capacity and recovery of pDNA for scale-up. For higher capacity, Millistak+® C0HC is a suitable pre-filter to Millipore Express® filters.
- Calcium chloride dosage screening showed a reduction in RNA and total DNA at greater than 0.4M concentration.
- Ultrafiltration/Diafiltration: Pellicon® cassettes with Biomax® 100-300kDa membrane show feasibility in concentration/buffer exchange of pDNA. Results show protein and DNA are both retained by the membranes. Larger cut-off 300kDa membrane is available for larger plasmids for higher flux/ passage.
- AEX chromatography: Natrix® Q membrane provides a high productivity method of purifying pDNA. Feasibility of binding 10 g/L pDNA was demonstrated at 10 MV/min or 6 sec residence time. Consistent flowthrough of RNA was observed for native and concentrated samples. Fractogel® DEAE provides an effective resin-based alternative.

The life science business of Merck KGaA, Darmstadt, Germany operates as MilliporeSigma in the U.S. and Canada.

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