Diagnostic studies to unlock process robustness at 1000 L scale of EPI-321, an AAV-based epigenetic therapy

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1. Abstract

EPI-321 is an epigenetic therapeutic designed to treat facioscapulohumeral muscular dystrophy (FSHD), a genetic muscle disorder that is estimated to be the second most common muscular dystrophy. Patients with FSHD commonly present with progressive muscle weakness and atrophy symptoms starting early in adulthood, often with facial and upper body weakness before progressing to the abdomen and lower body. EPI-321 addresses the root cause of FSHD by remethylating the D4Z4 region to suppress DUX4, by leveraging Epicrispr Biotechnologies' Gene Expression Modulation System ("GEMS") platform. GEMS allows us to regulate gene expression without making changes to the DNA. Our investigational product is delivered via a single adeno-associated virus vector, AAVrh74. Due to intravenous administration in adult patients and the substantial unmet medical need, high dosing requirements place significant demands on manufacturing yield. Here, we briefly outline the scale-up process to 1000 L, highlight key insights gained, and share data from investigative studies addressing process challenges.

To initially maximize manufacturing yields, we implemented engineered plasmids with a new transfection reagent and succeeded in boosting titer by upwards of 20-fold at small-scale. One of the primary challenges for upstream process developers is scaling the complexation of the plasmid DNA with the transfection reagent and eventual transfer to the cells. As batch volumes increase, the volumes of transfection complexes also grow, but chemically defined limitations, such as complexation time, remain constant. By characterizing complexation time, a critical process parameter, we established acceptable ranges and could provide more flexibility in transfer flow rates. To further understand how transfer flow rate impacts titer, we assessed different flow regimes (laminar, transitional, turbulent) during in-line mixing and final complex delivery. We next targeted key volume-dependent parameters such as agitation and aeration rate, which are difficult to keep constant with changing vessel geometries. These parameters directly affect transfection efficiency, but their more pronounced influence is on the cell culture environment which can be a critical determinant of run performance. To better characterize agitation rate, we assessed the impact of tip speed on productivity. We were able to synergistically apply the findings of these studies and successfully scale up to 200 L, accomplishing a 15-fold increase from historical titer. While these studies helped inform process ranges, complexation time, delivery volume, and agitation were not kept constant between scales due to operational restrictions. Here, we review the rationale behind manipulating these parameters at 1000 L scale, the observations from implementing these changes, as well as the studies conducted to characterize and evaluate how these factors contributed to the run's performance.

2. Platform

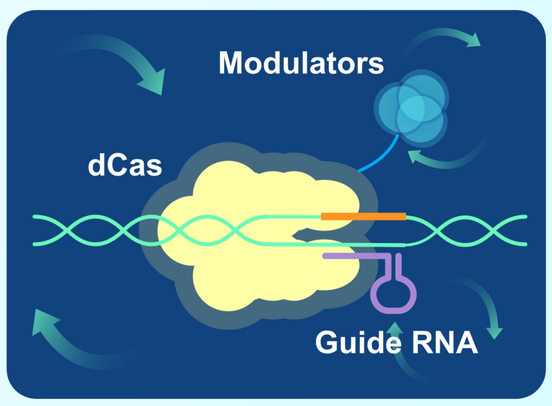

Figure 2: Epicrispr Biotechnology's GEMS Platform. System comprised of gRNA, dCas, and modulator proteins.

GEMS: ***G***ene ***E***xpression ***M***odulation ***S***ystem

- The **guide RNA** (gRNA, purple) provides specificity for the epigenetic editing. It's the "genome GPS" for the GEMS system
- The **nuclease "dead" Cas protein** (CRISPR-associated protein, yellow), fused to the modulator, binds to the gRNA at the target site. It does not cut the DNA
- The **modulator proteins** (blue) are engineered to modify the epigenome and either activate or repress the targeted, nearby gene

3. Background

Historical EPI-321 Process 1A titers in E+10 vg/mL range made it challenging to support doses for systemic administration while minimizing COGs. Insufficient scaling from bench to production-scale was observed; therefore, it was essential to optimize titers to ensure commercial success and mitigate scaling losses. Screening an engineered Helper and RepCap plasmid against next-generation transfection reagents (TRs) helped us select a transfection strategy that increased productivity by 10x. Process 1B was then scaled to 50, 200, and 1000 L; however, inconsistent performance was observed.

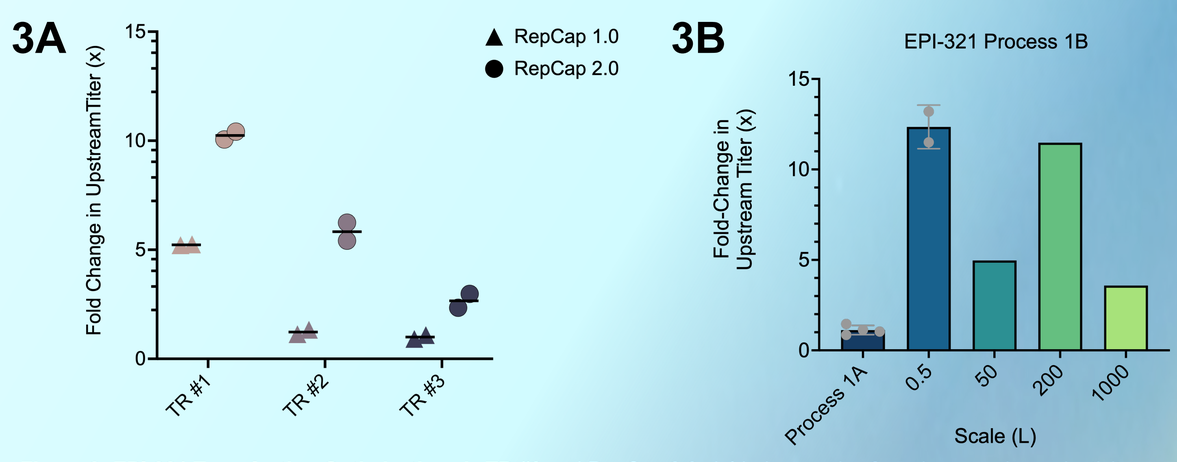

Figure 3: EPI-321 Transfection Optimization. A. TR #2 and RepCap 2.0 yielded upwards of ~5x increase in productivity compared to platform reagent (TR #3). High clarified lysate titers suggest synergistic effects between engineered plasmids and optimized reagent. Small scale purification data identified TR #2 as the superior reagent due to impurities observed in TR #1 drug substance. **B.** Upstream titers across increasing scale show conflicting results. Data suggests looking for similarities between 0.5 and 200 L, and 50 and 1000 L.

4. Cell Counting Optimization

Upon investigation of all parameters, it became evident that our proprietary cell line was susceptible to aggregation, and this was mostly dependent on scale. Due to variable aggregate amounts, the growth profile across productions was inconsistent and introduced heterogeneity within the cell culture. A lysis-mediated cell counting method was implemented to bypass this symptom.

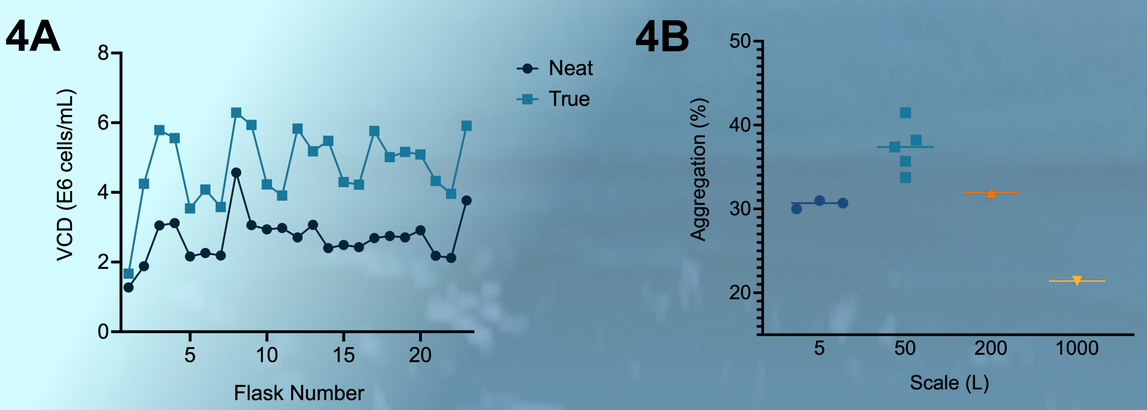

Figure 4: Cell Line Counting and Aggregation. A. Neat vs. lysis-mediated cell counts from ongoing seed train at various flask scales (30, 60, 300, 1200 mL working volumes). 1.77x increase in cell density observed between counting methods. **B.** Variable levels of aggregation seen across production scales. Data suggests that aggregation (%) may be closely related to other volume-dependent parameters such as agitation and provides justification to look at inter-parameter relationships.

5. 1000 L Investigation

Scaling of EPI-321 Process 1B from bench to 200 L posed minimal challenges beyond optimization of transfection strategy. However, when projected to 1000 L with volume-dependent parameters held constant, several operating ranges were at or beyond their limits. To maximize the likelihood of success at this scale, targeted adjustments were implemented to several parameters. The following table summarizes the changes made, along with brief rationale:

Parameter	200 L 🟢	1000 L 🔴	Reason/Justification
Agitation Rate (m/s)	1.2	1.5	*Agitation strategy to match across scales by power input increased tip speed*
True Transfection Density* (E6 cells/mL)	5	7	*Deviation observed in 1000 L, exacerbated effect due to aggregation*
Double-Bolus Transfection (Y/N)	N	Y	*To keep complexation time under cliff of 45 minutes, a double-bolus, sequential transfection method was used*
Complexation Time (min)	21	43	*Due to chemically-defined limitations of the transfection reagent, PEI:DNA cocktail could not be pumped faster than 1.1 LPM; therefore, extending complexation time*

*Projected using average correction factor of 1.77x between neat and true cell counts in Figure 3a.

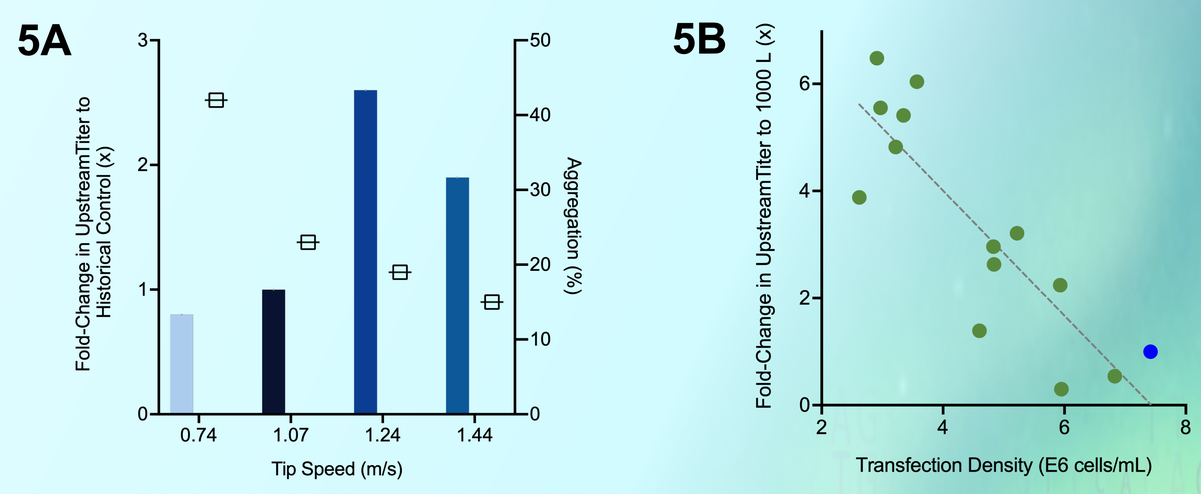

Figure 5: 1000 L Investigational Supporting Studies. A. Study results depict a positive relationship between tip speed and productivity. In contrast, the data shows a negative relationship between tip speed and aggregation; therefore, indicating that higher aggregation is detrimental to productivity. Experiment suggests that tip speed did not cause poor performance at 1000 L. Experiment executed at 5 L scale. **B.** Graph portrays a moderate to strong effect of transfection density on upstream titer. Blue dot represents 1000 L production and fits experimental model. High transfection density can lead to upwards of ~90% loss in titer. Experiment(s) executed at 2 and 5 L scales.

6. Diagnostic Study

ID	Tip Speed (m/s)	Double Bolus (Y/N)	Complexation Time (min)	Transfection Density (E6 cells/mL)
V1	1.5	N	30	3
V2	1.5	Y	30	3
V3	1.5	Y	45	3
V4	1.5	Y	45	4.2
V5	1.5	Y	45	5.2

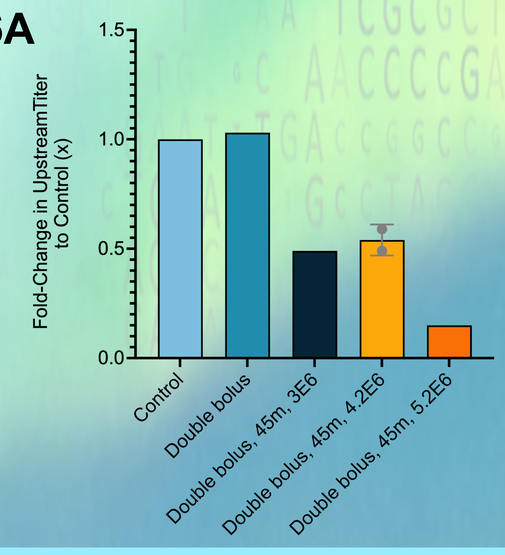

Figure 6: Additive 1000 L Diagnostic Study. A. Stepwise experimental design used, starting with a control condition representing optimal operation and sequentially adding one parameter at a time. Design allows assessment of individual contribution and any synergistic detrimental effects. Results indicate that extended complexation time reduces titer by ~50%. A defined transfection density window of 3 to 4.2E6 cells/mL was identified, beyond which cell growth led to productivity losses up to 90%. Experiment executed at 5 L scale.

7. Summary

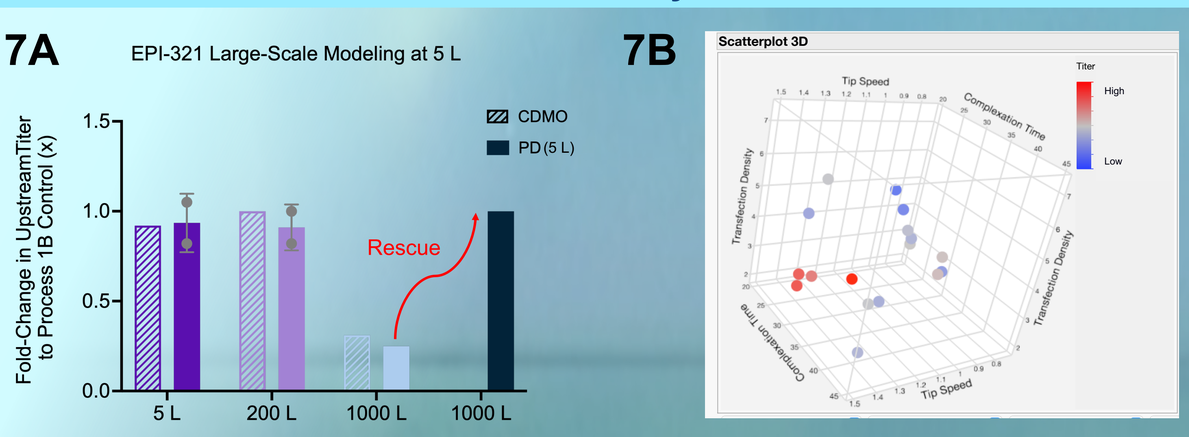

Figure 7: EPI-321 Upstream Modeling. A. Scaledown models successfully established for 200 and 1000 L processes. 1000 L PD production modeled at 5 L shows comparable performance, while "rescued" 1000 L (with accurate density) achieves consistency across scales. **B.** Multivariate analysis via JMP identified safe ranges for transfection density (2-4E6 cells/mL), tip speed (>1.2 m/s), and complexation time (30m) for potential implementation in Process 2.

Conclusions & Future Work

- ✓ Largest effect was linked to transfection density, with lysis-mediated cell counts mitigating variability from cell clumping and enabling tighter process control
- ✓ TR #2 complexation time is a scaling constraint beyond 1000 L (ceiling at 50% productivity), highlighting the need to explore more robust alternatives for Process 2
- ✓ Next steps include scaling Process 2 to 50 L

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CRISPR-Based Epigenetic Upregulation of Endogenous Utrophin as a Mutation-Agnostic Therapeutic Strategy for Duchenne Muscular Dystrophy
Abhinav Adhikari
Fibes 2, October 8, 14:00-15:30