

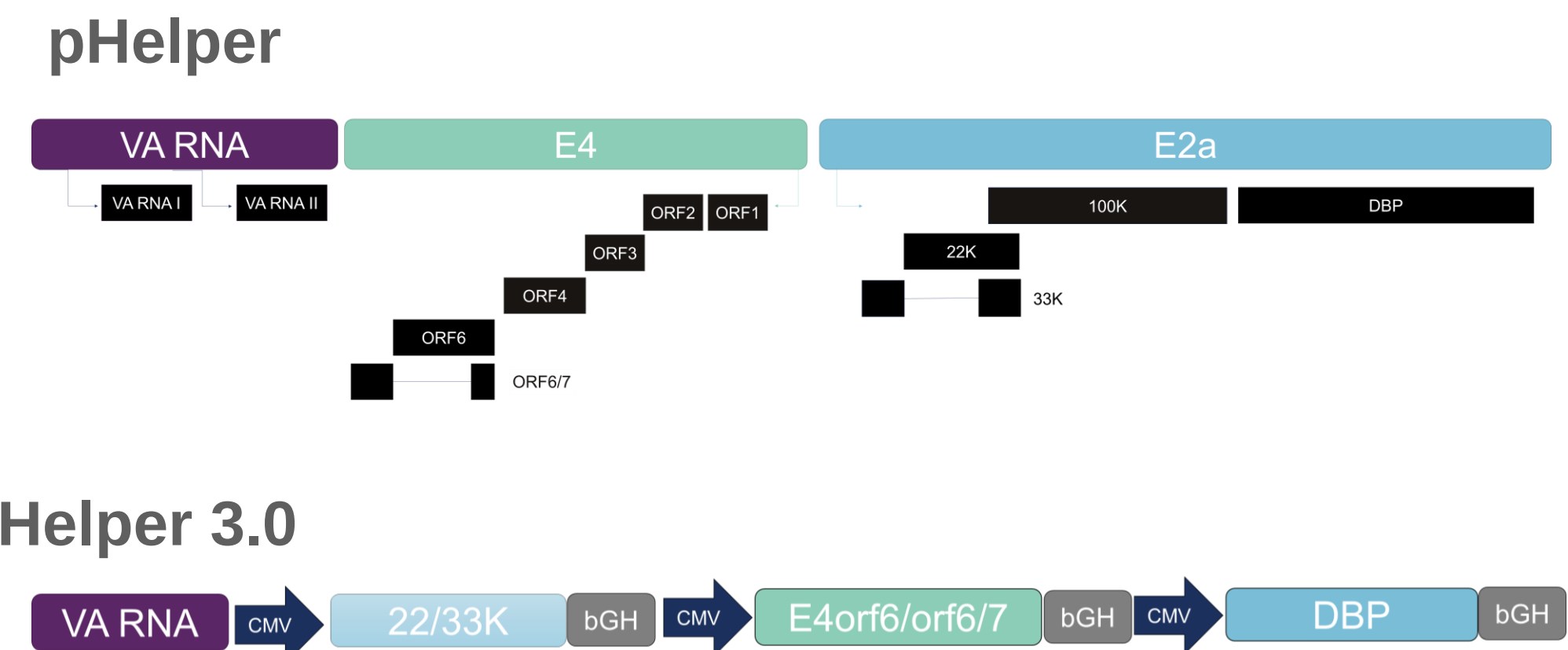
Enhancing AAV Production Yield Utilizing An Engineered Adenoviral Helper Plasmid

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Introduction

A Helper plasmid is critical for adeno-associated virus (AAV) vector production using transient transfection. While the traditional, native adenovirus derived Helper plasmids encode functional helper genes, E2a, E4 and VA RNA to support AAV replication and gene expression, native adenovirus gene regulation does not necessarily provide the most optimal conditions for AAV production. To optimize an engineered helper plasmid, DNA binding protein (DBP), L4 22K/33K, E4orf6/7, and VA RNA were evaluated independently as separate plasmids, and expression of each was assessed to determine their impact on AAV vector production. Based on these insights, our Helper 3.0 was designed to provide helper gene expression in an optimal manner to enable more efficient AAV production.



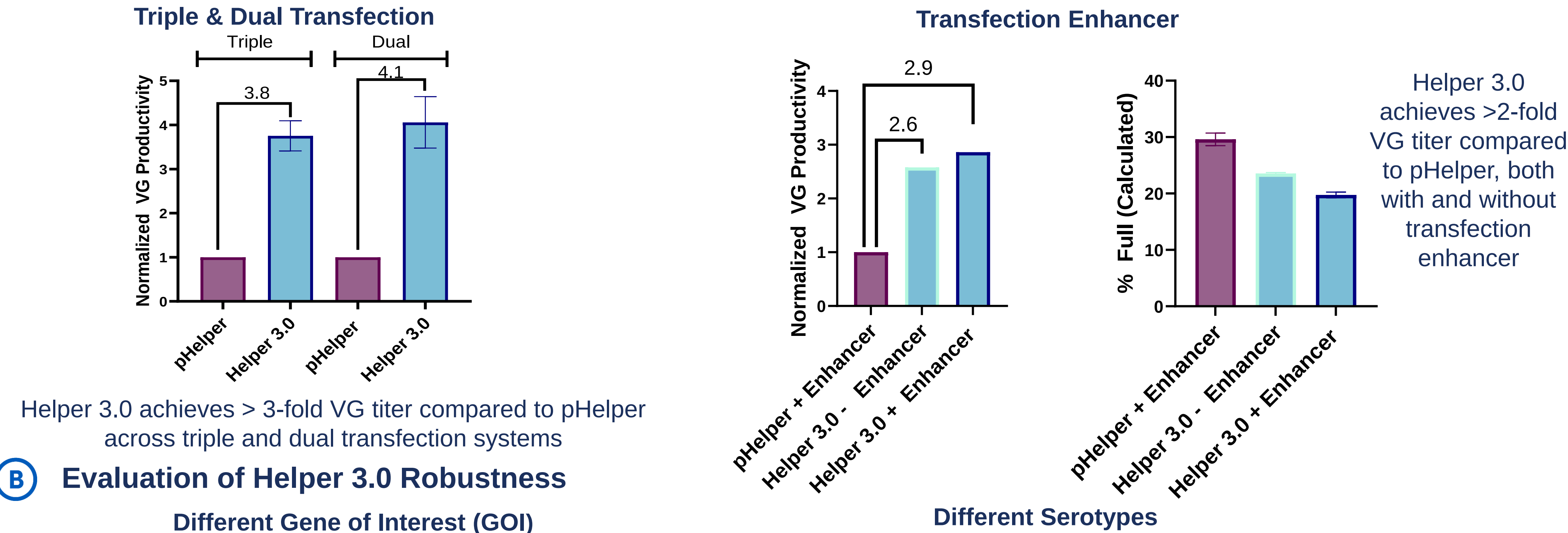
We employed a platform development approach to evaluate Helper 3.0 for its ability to enhance vector genome (VG) titer productivity within our recombinant adeno-associated virus (rAAV) transfection system. Helper 3.0 was evaluated across triple and dual transfection platforms using multiple genes of interest, capsid serotypes, plasmid ratios, and scaled up from shake flasks to bioreactors. Performance was measured based on vector genome titer and product quality to determine its capacity to deliver robust, scalable improvements over the native pHelper plasmid.

Methods

Initial experiments were conducted in 125mL shake flasks with a 50mL working volume using VPC 2.0 cells (Thermo Fisher). Shake flasks were seeded at 2×10^6 cells/mL in Expi293 media (Thermo Fisher). All experiments utilized the OXB triple and dual plasmid system (GOI+RepCap), containing a CMV-GFP/Luciferase genome and the AAV9 capsid. Subsequent plasmid ratio testing was performed in shake flasks to identify conditions that maximize productivity. The optimized transfection conditions were then scaled to a 2L bioreactor, and harvested material was further purified using affinity chromatography. Vector genome (VG) and capsid titers were analyzed by ddPCR and ELISA (Progen), respectively. Sedimentation velocity analytical ultracentrifugation was used to quantify the relative abundance of empty, partially filled, and full capsids of affinity product.

Results

A Helper 3.0 Platform Performance



B Evaluation of Helper 3.0 Robustness

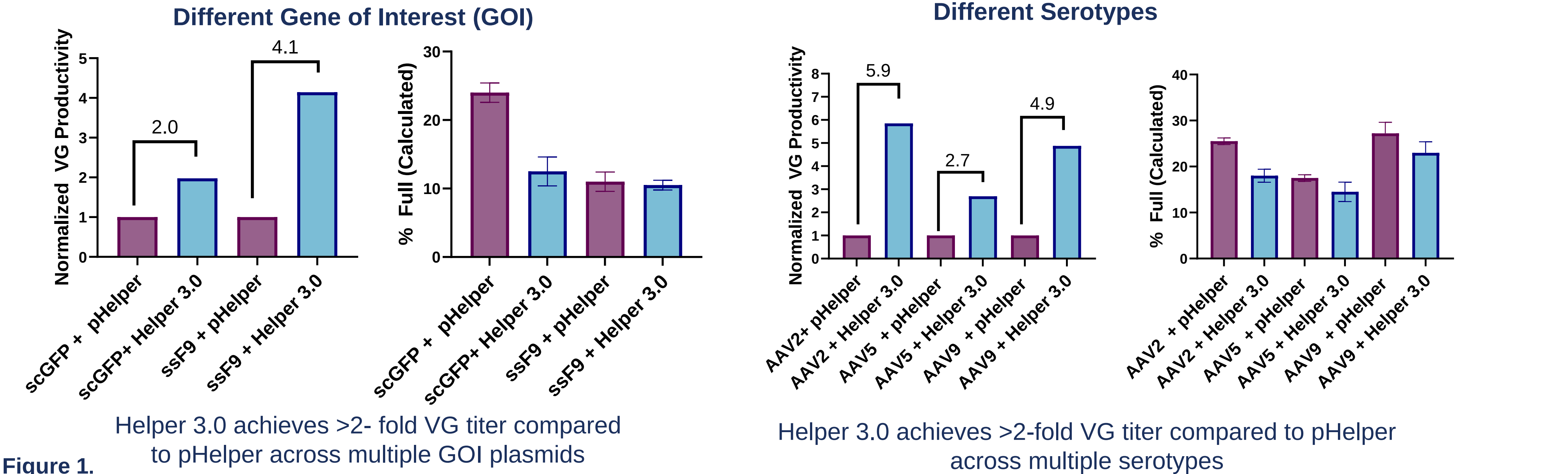
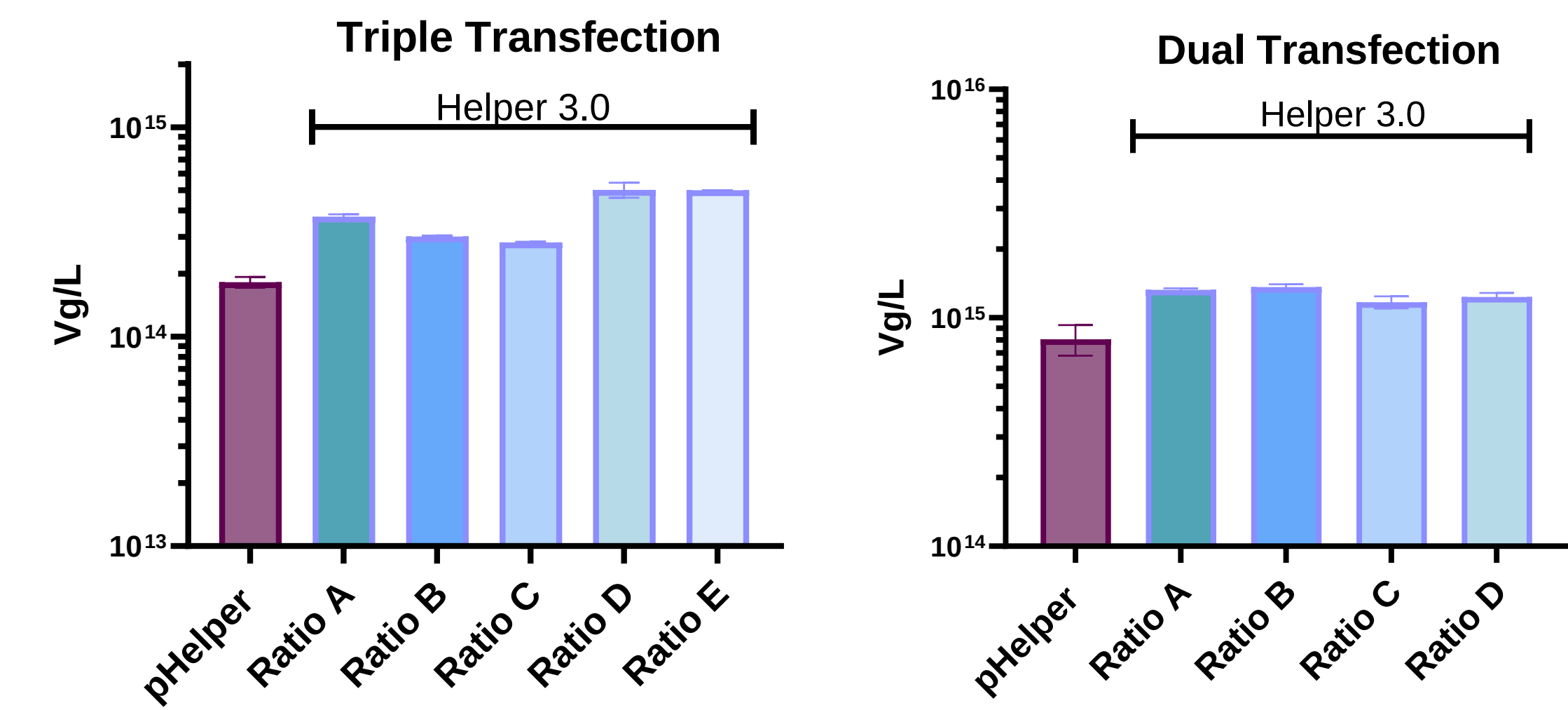


Figure 1.

AAV vector genome (VG) titers and calculated % full capsids in crude lysate were compared between the original pHelper and the engineered Helper 3.0. Helper 3.0 consistently achieved over a 2-fold increase in VG titer, with a modest reduction in % full capsids relative to the native helper plasmid (A-B). This trend was observed across (A) triple and dual plasmid transfection and with and without transfection enhancers and using (B) different genes of interest (self-complementary (sc) GFP and single stranded (ss) factor 9 (F9)). Evaluation of (C) AAV2 and AAV5 capsids with OXB Helper 3.0 demonstrated similar patterns, indicating that the VG increase is not serotype-specific.

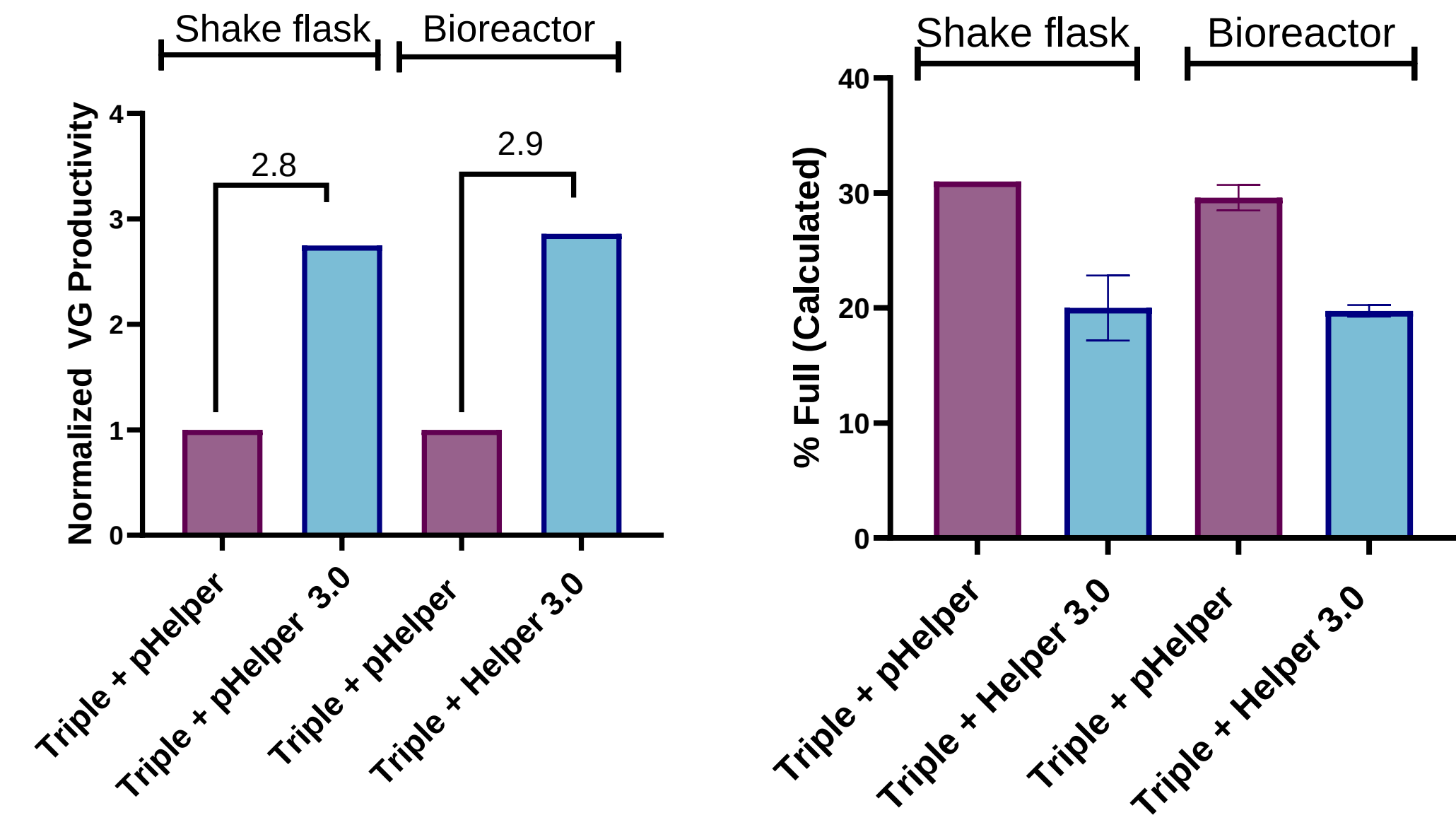
Plasmid Ratio Screening



Plasmid ratio D was selected as optimal for triple transfection. Dual plasmid ratios showed comparable performance

Figure 2. Comparison of AAV vector genome titers in crude lysate from shake flask plasmid ratio screening of dual and triple plasmid systems. All tested ratios consistently produced >1.5-fold increase in vector genome (VG) yields compared to the native pHelper plasmid, demonstrating reproducible productivity. For triple transfection, plasmid ratio condition D was selected based on overall productivity and cost-efficiency and advanced for evaluation at the 2L bioreactor scale. Dual plasmid ratios exhibited comparable performance across conditions.

C Upstream Scale-Up Performance



Helper 3.0 successfully scaled from shake flasks to small-scale bioreactors, achieving 3-fold titer increase

D Affinity Purified Product Quality

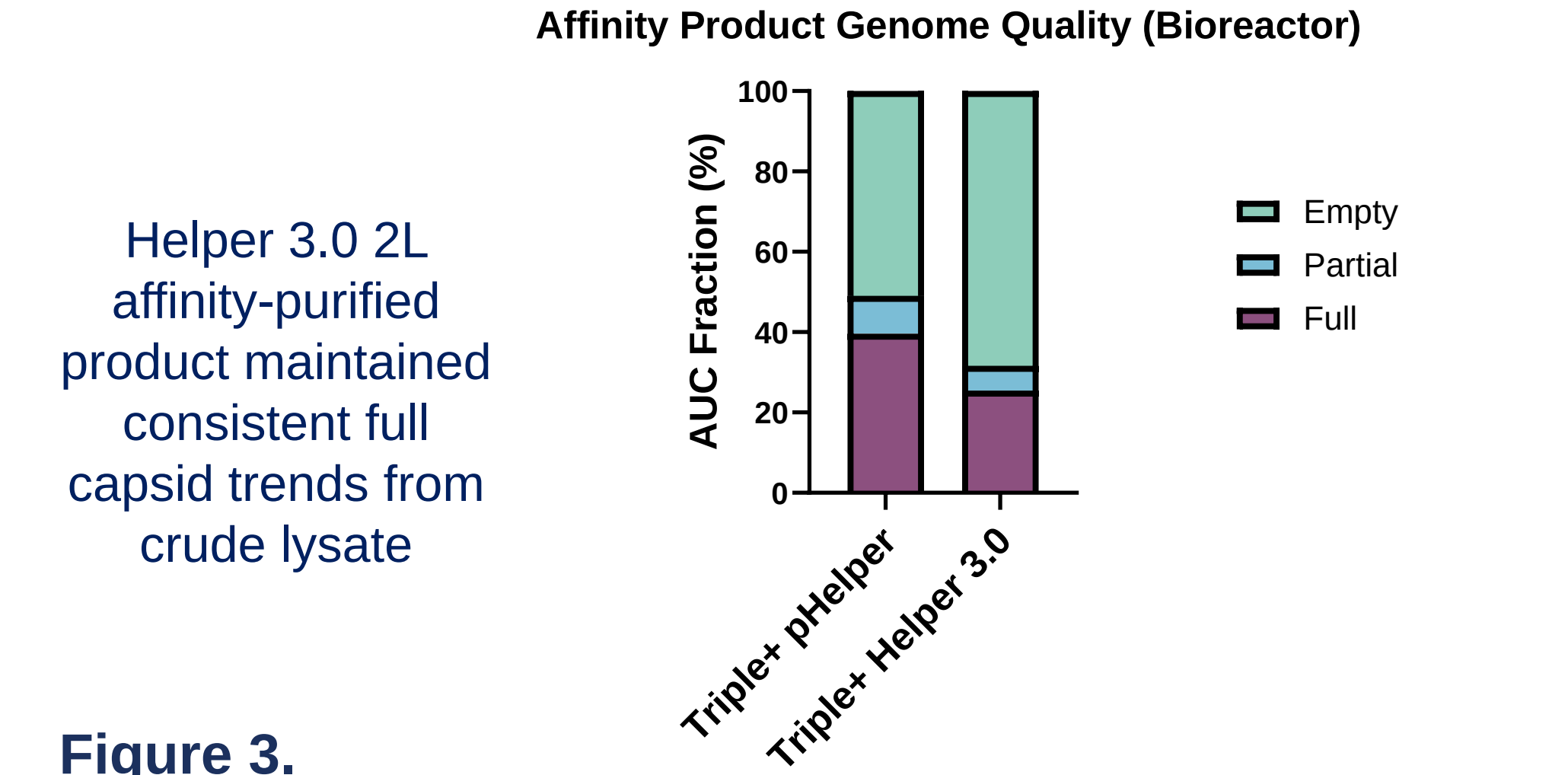


Figure 3.

AAV vector genome (VG) titers and calculated % full capsids in crude lysate were compared between the native pHelper and engineered Helper 3.0. (C) Helper 3.0 maintains enhanced productivity, achieving ~3-fold titer increase with consistent % full capsids from shake flasks to 2L bioreactors, demonstrating scalable and reproducible performance. Helper 3.0 (D) 2L affinity purified product genome quality showed consistent % full trends from the crude lysate stage, with most capsids being empty and approximately 25% calculated as full.

Conclusion

Engineering the native helper plasmid into an optimized helper design enhances rAAV production across serotypes, increasing vector genome and capsid yields reproducibly without affecting affinity product quality. Helper 3.0 delivers robust, scalable improvements in transfection systems compared to native helper plasmid for efficient production.

