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Simplifying Progress

Integrated Plasmid Engineering and AAV Manufacturing Process a Unified Approach from Lab to Clinic

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Plasmids are essential tools in basic research, diagnostics, and medical applications. Their versatility, ranging from traditional cloning and expression vectors to highly optimized clinical-grade DNA constructs, makes them suitable for both fundamental studies and advanced therapeutic strategies, including gene and cell therapies as well as RNA and DNA vaccines. Plasmids used in current biomanufacturing differ fundamentally depending on whether they are designed for RNA, adeno-associated virus (AAV) or lentiviral (LV) production. Indeed, certain specific nucleotide sequences, such as a poly(A) tail, inverted terminal repeats (ITRs) or long terminal repeats (LTR) that are present in plasmids for mRNA, AAV and LV manufacturing can become challenging for their production and/or purification. By optimizing plasmid design and plasmid manufacturing, researchers and developers can enhance the safety, efficacy, and specificity of biotherapies, ultimately improving treatment outcomes.

Sartorius has established an integrated service platform that enables the design, construction, production, and purification of all types of plasmids used in advanced therapies. The commercialization of an optimized pHelper plasmid dedicated to the production of AAV with improved quality in terms of empty/full ratio and infectivity has involved three selection steps made possible thanks to the e-Zyvec® assembly technology, as well as an adaptation of the plasmid production method.

Plasmid engineering with e-Zyvec® DNA assembly technology

Sartorius has developed a unique proprietary DNA assembly technology (e-Zyvec® technology) enabling to assemble any DNA sequences in the desired order and orientation, at the nucleotide. Thanks to its flexibility and accuracy, the technology simplifies the generation of any type of new plasmid, from the easiest to the most sophisticated (Figure 1).

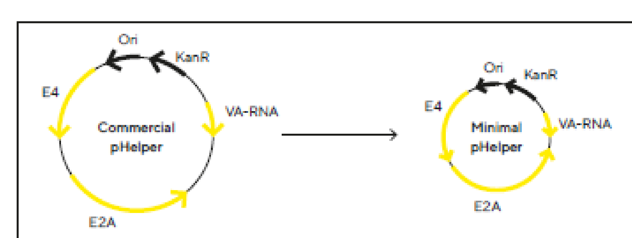
Figure 1: e-Zyvec® DNA assembly technology. Based on the desired plasmid specifications defined using our online design platform, linear DNA fragments are built and assembled, seamlessly, in a single reaction.

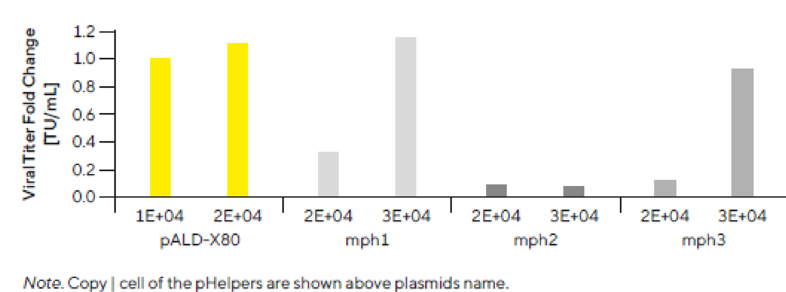
Building an optimized AAV pHelper

The e-Zyvec® DNA assembly technology has been exploited to explore the synergies of multiple genetic DNA features modularly assembled to generate a set of new AAV Helper plasmids (pHelpers). Comparison of the biological activity of the several AAV pHelper prototypes (57 in total) led us to identify the optimal architecture which outperforms existing helper plasmids under all tested bioproduction conditions. The plasmid design strategy leading to the generation of an optimized AAV pHelper is presented in Figure 2.

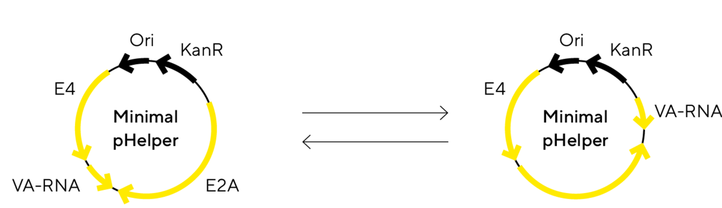
1. **Removing non essential elements**

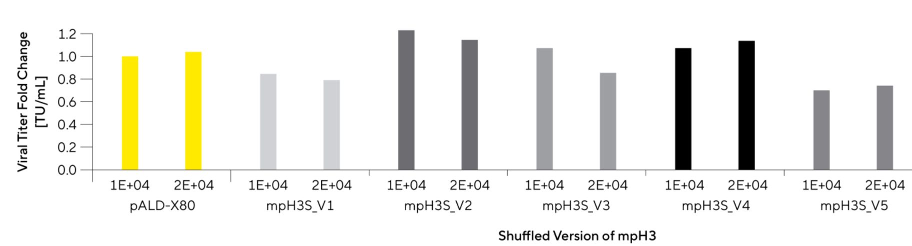
Construction of a Minimal pHelper (mpH)
▪ 19 constructions tested, 3 series





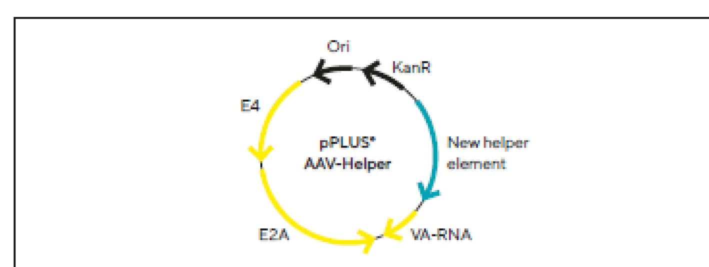
2. **Shuffling DNA features**





3. **Adding new helper elements**

Identification of New Sequences to Add to the pHelper to Boost rAAV Production
▪ 29 constructions tested, 5 series



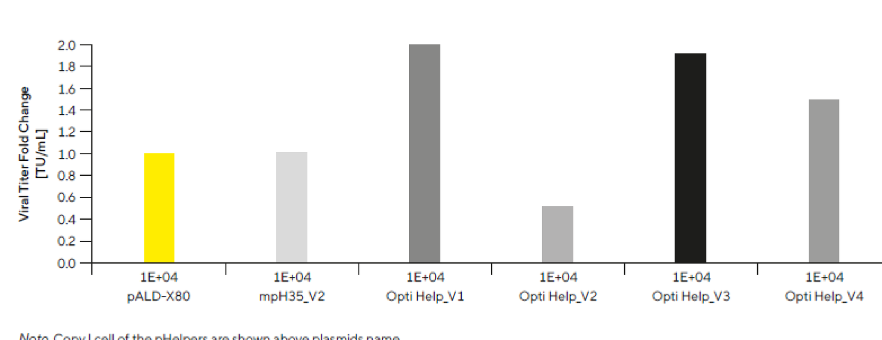


Figure 2 : Plasmid design strategy for generating an optimized AAV pHelper. 1) Removing non essential elements. 19 constructs were engineered by removing non essential elements from a standard pHelper plasmid. This step led to the generation of a minimal pHelper. 2) Shuffling DNA features. Plasmid architecture of the minimal pHelper plasmid was challenged by shuffling DNA features. 9 new constructs were generated. 3) Adding new helper elements. Addition of new DNA sequences in the plasmid architecture was assessed on AAV production yield. During this last step, 29 new constructs were generated.

Optimization for manufacturing of AAV pHelper plasmid

Features of the selected pPLUS®-AAV helper plasmid:

- Size: 13.3 kb
- Sequence: Contains genetic elements that promote AAV production, with a positive impact on F/E ratio & infectivity

Step 1: Evaluating standard batch fermentation at 50 L scale

pPLUS®-AAV helper plasmid production was performed using our standard batch fermentation approach that consists of an initital fermentation phase aimed to generate biomass, followed by a second phase induced with a temperature shift to accelerate replication rate and increase plasmid production yield. As shown in Figure 3, we obtained a low plasmid yield with a yield before induction (BI) of 150 mg/mL to a harvest yield (H) of 200 mg/mL, corresponding to a H/BI yield ratio of 1,3.

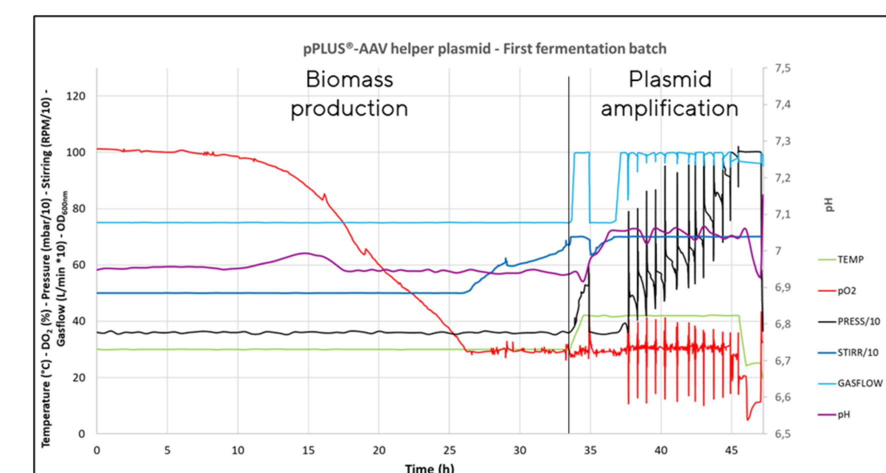


Figure 3. Fermentation profile using standard protocol

Step 2: Process optimization of pPLUS®-AAV helper plasmid production at 5L scale

Process optimization of pPLUS®-AAV helper plasmid production was performed at smaller scale of 5L. Four additional fermentation protocols were evaluated (table 1). Plasmid specific yield was measured after temperature shift for the four fermentation conditions. As shown in Figure 4, in comparison to the standard process (F03), a softer (F05) or shorter (F06) induced temperature shift led to improved yield at harvest, respectively 461 mg/mL and 359 mg/mL. However, as shown in Table 1, a higher gDNA content due leading to cell toxicity was observed when plasmid replication was boosted by induced temperature shifts. Removal of the plasmid replication phase led to a reduced gDNA content after induced temperature shift in fermentation F04 strategy.

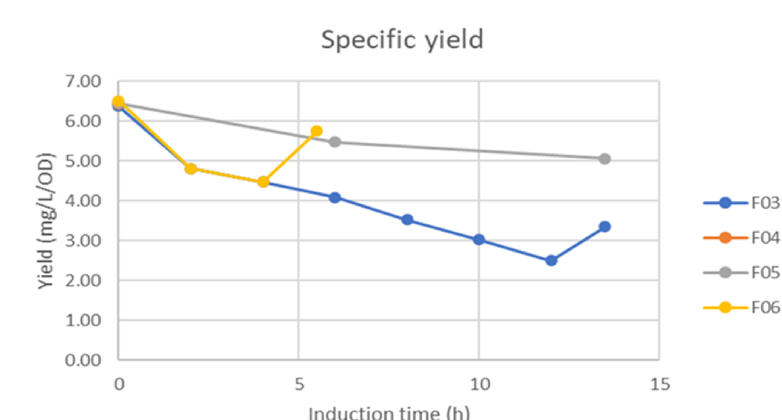


Figure 4: Plasmid specific yield recorded after T° shift. Cell toxicity was observed when plasmid replication was boosted by induced temperature shifts. The selected fermentation protocol omits this amplification phase while achieving a sufficient productivity of 300 mg/L and a low gDNA content of 0.37% before any purification.

Fermentation #	F03	F04	F05	F06
Conditions	Standard process	No replication phase	Softer induction	Shorter induction
Yield before induction (BI) mg/L	319	321	316	322
Yield at harvest (H) - mg/L	268	321	461	359
Ratio yield H/BI	0,84	N/A	1,46	1,11
gDNA content BI - %	0,38	0,37	0,36	0,35
gDNA content (H) - %	1,22	0,37	1,14	0,77

Table 1. Plasmid recovery yield and gDNA content recorded before induction (BI) and at harvest (H).

General Conclusions

pPLUS®-AAV plasmid production		
Steps	**pPLUS®-AAV helper plasmid**	**Tailor made pPLUS®-AAV Rep/Cap & pPLUS®-AAV transgene plasmids**
Plasmid design & engineering	pPLUS®-AAV helper plasmid optimized for improving viral particles quality and infectivity	Tailor made pPLUS®-AAV Rep/Cap and AAV transgene plasmids helper plasmids using e-Zyvec® assembly technology
Plasmid manufacturing	Fermentation conditions specifically adapted to pPLUS®-AAV helper plasmid features	Fermentation conditions adapted to plasmid features of Transfer (ITR) and Rep/Cap plasmids