

Abstract

Synthesis and propagation of plasmid DNA molecules with complex sequence motifs is an established pain point in manufacturing genetic medicines. Herein we report the use of a groundbreaking enzymatic DNA synthesis platform from Ansa Biotechnologies in conjunction with Novel Bio’s in-house DNA assembly workflow to reliably synthesize and produce complex plasmid DNAs used in the production of viral vectors for gene therapy.

Introduction

Genetic medicines require the introduction of novel genetic information into cells to either endow the cells with a new function (such as programing T cells to target specific types of cancer in the case of CAR T therapies) or to replace a damaged gene with a corrected copy to restore proper cellular function. While there are different approaches to introducing novel genetic information into cells, plasmid DNA is the universal starting point for all of them.

Novel Bio is a company pioneering new technologies for plasmid DNA production, with a focus on genetic medicines. We are developing new host organisms for plasmid production such as our NBx Platform™, as well as cell-free technologies for scaleup of supercoiled plasmid DNA.

In order to produce the desired plasmid, the first step is to generate the necessary DNA components, which for novel sequences is accomplished via gene synthesis technologies. Challenges arise in that certain DNA sequences are extremely difficult to synthesize using traditional methods. For example, regulatory sequences commonly referred to as untranslated regions (or UTRs for short) often contain repetitive sequence motifs, large swings in GC content, and/or sequences that form secondary structural elements. The presence of such sequence motifs interferes with most gene synthesis approaches, making the synthesis of such DNA fragments extremely difficult if not impossible.

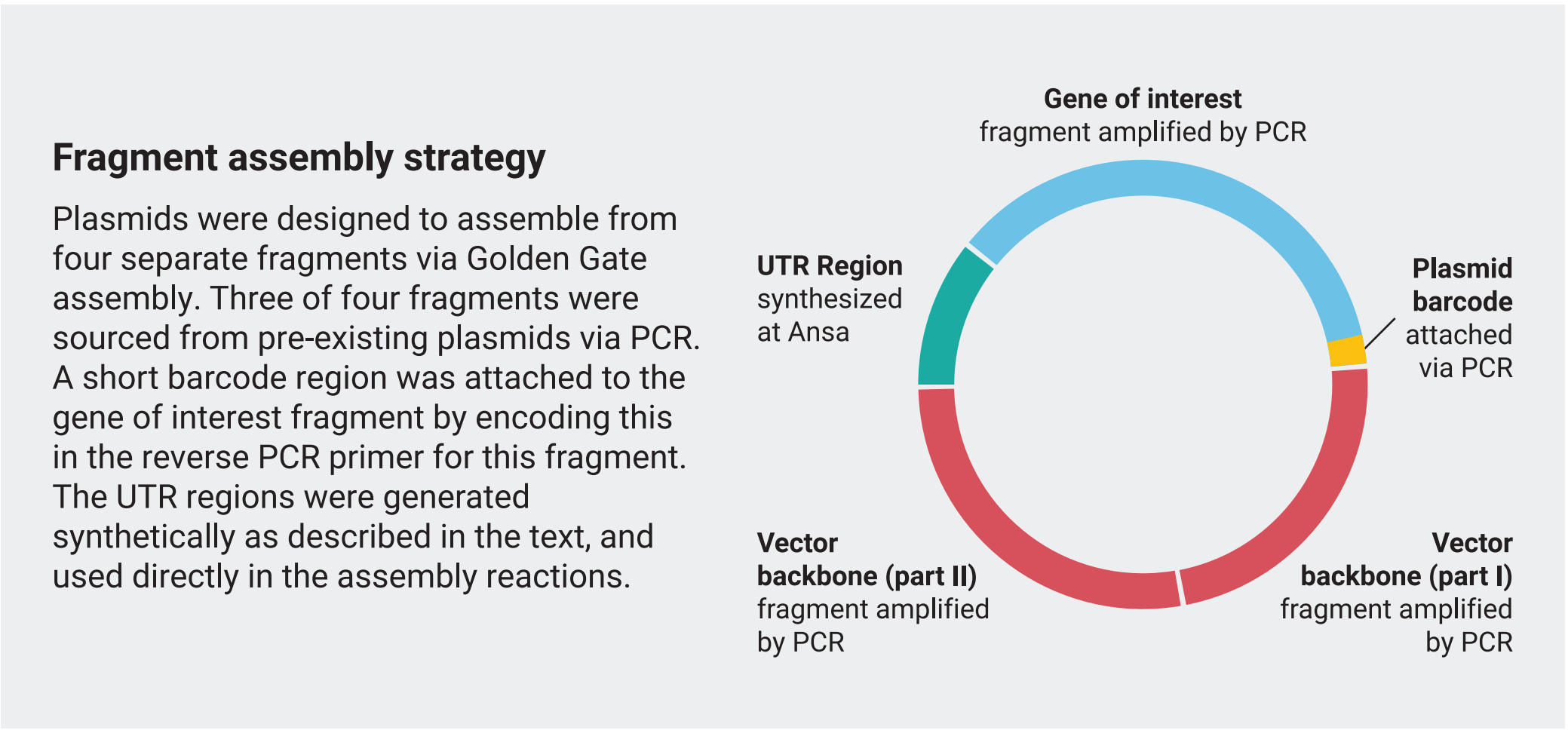
Furthermore, even if one can successfully synthesize the DNA, the DNA parts need to be assembled into the final plasmid molecule. It is common for complex sequences to undergo undesired alterations or recombination events during molecular cloning and process of scaling up the plasmid.

In a recent project, we were tasked with synthesizing, cloning, and scaling up a variety of gene therapy plasmids containing complex UTR sequences. In order to successfully deliver on this project, we leveraged two capabilities:

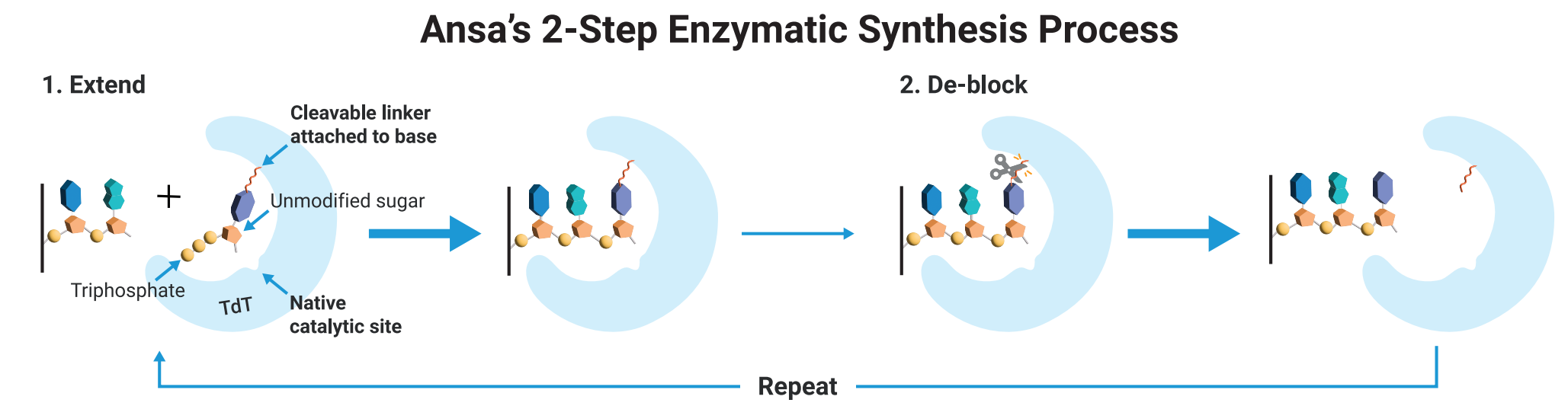
- 1. A proprietary enzymatic DNA synthesis technology developed by Ansa Biotechnologies¹.
- 2. Novel Bio’s in-house DNA design, assembly, and scaleup workflows, optimized for the high-fidelity production of complex plasmid DNA molecules.

DNA Synthesis & Assembly

Using in-house computation tools available at Novel Bio, we designed an assembly strategy for 51 gene therapy plasmids, each differing in the UTR sequence providing regulatory control over the gene of interest. The plasmid assembly strategy was designed to leverage as much pre-existing sequence as possible, which could be isolated from existing plasmids via PCR (to save time and cost). While we were able to source the plasmid backbone and gene of interest for the constructs via PCR from existing plasmids, the novel UTR sequences were not available for an existing source, and therefore needed to be generated via gene synthesis.



There are many commercial vendors that offer gene synthesis services, mostly relying on chemical synthesis of short oligonucleotides which are then pooled and stitched together in a PCR-like enzymatic reaction. This contrasts with Ansa’s enzymatic DNA synthesis approach, where the entire DNA fragment is synthesized as a single DNA strand with each base added to the growing chain via an enzymatic process using polymerase-dNTP conjugates¹, followed by an amplification process to generate double-stranded DNA. This innovative approach overcomes many of the synthesis limitations imposed on traditional approaches.



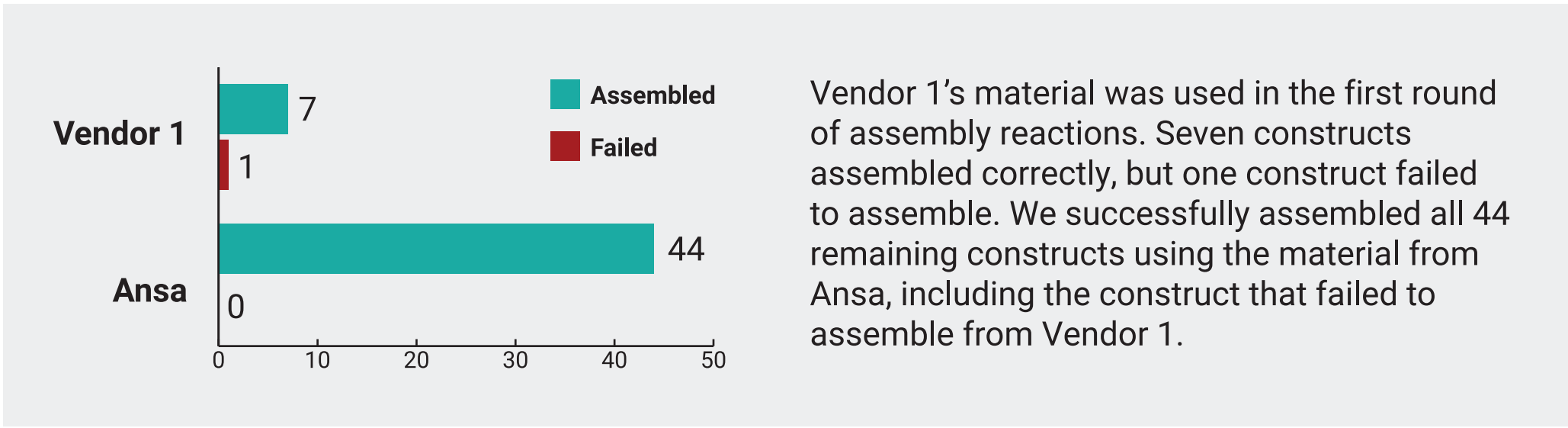
We submitted 51 UTR sequences for complexity screening to three commonly used DNA synthesis vendors, as well as to Ansa Biotechnologies. As can be seen from Table 1, the majority of the UTR sequences were rejected outright by most vendors during complexity screening, though 100% were accepted for synthesis by Ansa.

TABLE 1				
	Vendor 1	Vendor 2	Vendor 3	Ansa
Accepted	1	15	13	51
Accepted, but flagged as complex	7	1	0	0
Rejected	43	35	38	0
Total	51	51	51	51

TABLE 1: Summary of complexity evaluation of DNA fragments from three widely used commercial DNA synthesis vendors as well as Ansa Biotechnologies. Sequences in “ACCEPTED” row met the criteria set by each vendor’s complexity algorithms, and are predicted to be amenable to synthesis. Sequences in the “ACCEPTED, BUT FLAGGED AS COMPLEX” row are flagged by the respective algorithm as containing sequence motifs that put the synthesis at risk, but the vendor is still willing to attempt the synthesis. Sequence in the “REJECTED” row are flagged as containing challenging sequence motifs that exceed the complexity threshold set by each vendor and are predicted to fail during synthesis. As such, vendors refuse to attempt the synthesis of these fragments.

Vendor 1 was able to generate material for all 8 constructs that passed Vendor 1’s internal QC metrics, which included evaluating fragment size by capillary electrophoresis, as well as fragment yield. In parallel, Ansa generated material that passed Ansa’s internal QC metrics for all 51 constructs, which included fragment sequence (by NGS), and fragment yield.

With DNA fragments in hand, we proceeded to plasmid assembly. The UTR sequences were designed to assemble with the plasmid backbone and gene of interest via Golden Gate assembly². As these particular viral vector plasmid designs are known to be extremely recombinogenic, resulting in plasmid truncations as a result of recombination between the inverted terminal repeats contained in the backbone, we used a proprietary scale up workflow to generate each of these plasmids without undesired modifications. Following assembly and cloning, we selected 2-6 clones of each construct for full plasmid sequencing.



Discussion & Conclusions

Genetic medicines hold great promise as the next wave of biomedical innovation, yet they will never realize their full potential until manufacturing issues can be adequately addressed. Here we have shown how innovations in DNA synthesis and assembly enable the routine production of plasmid designs that would have been difficult, if not impossible, to generate using conventional techniques. These innovations include the use of Ansa’s enzymatic DNA synthesis platform in conjunction with Novel Bio’s in-house workflows for plasmid DNA production. The use of these technologies enabled us to seamlessly deliver on a complicated gene therapy project.

Without this capability, this project would have become considerably more complex and costly. Relying on typical vendors, if a sequence is rejected, one is left to try to break the desired synthetic gene into multiple smaller fragments (hoping that the smaller fragments have a low enough complexity score to be accepted), which then increases the number of fragments that need to be stitched together via plasmid assembly to give the final product, thus decreasing the likelihood that a successful assembly will take place (and necessitating screening of more clones for a correct sequence). In cases where even the smaller fragments are rejected, the last resort is to break the gene up into overlapping synthetic oligos, and attempt to assemble the gene via a PCR-like reaction. For complex sequences this approach usually requires a significant amount of troubleshooting and optimization to identify conditions that yield the desired DNA fragment, significantly increasing the time and cost of the project.

REFERENCES:

1. Palluk, S., Arlow, D., de Rond, T. et al. De novo DNA synthesis using polymerase-nucleotide conjugates. *Nat Biotechnol* 36, 645–650 (2018).

2. Engler C, Kandzia R, Marillonnet S (2008) A One Pot, One Step, Precision Cloning Method with High Throughput Capability. *PLoS ONE* 3(11): e3647.