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Low Metabolic Burden Plasmid Production

Nature Technology's DNA Manufacturing Platform Tackles Instability and Toxicity Issues

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DNA vaccines, wherein a new antigen is inserted into a validated vector, have tremendous potential for deployment in pandemic applications as they can be rapidly produced in a validated, fermentation-purification process.

For this application, it is essential that the vector and fermentation process function with a variety of different antigen genes. Antigen genes may be unpredictably toxic or otherwise low yielding in standard fermentation processes, however. Careful consideration of host strain, plasmid, and production process factors have led to innovations that allow Nature Technology to routinely achieve high plasmid yields, even with plasmids known to be toxic or unstable when using standard production methods.

Several studies on various plasmid host strains indicate that plasmid yield and quality are significantly affected by the choice of host strain. Together, these studies also demonstrate that plasmid production in shake flasks is poorly predictive of plasmid production in fermentation, and that a strain's performance can be affected by the fermentation process. In general, *recA*, *endA*, and *relA* mutations in a host strain are beneficial for plasmid production. We have found that DH5a is a good host strain to use as it consistently produces high-quality plasmid DNA, even with difficult plasmids.

Plasmid copy number within a strain is largely set by vector-intrinsic factors. Gene therapy or DNA vaccine plasmids typically contain either the pUC or pMM1 temperature-sensitive origin of replication. This temperature sensitivity is especially useful for inducing high-yield plasmid production in fermentation. Standard ROP-minus replication origins derived from pBR322 have also been used, but have much lower copy number than pUC vectors, and consequently lower fermentation yields.

Antibiotic-Free Selection

High-copy plasmids can impose a metabolic burden on their hosts, giving plasmid-free cells an advantage in culture. Consequently, even a small number of plasmid-free cells can quickly overtake an entire fermentation in the absence of selection. Plasmid-free cells can be controlled by using a selectable marker in the plasmid. Antibiotic-resistance markers, typically kanamycin resistance (KanR), allow selective retention of plasmid DNA during bacterial fermentation and are the most commonly utilized selectable markers.

To ensure safety, however, regulatory agencies recommend elimination of antibiotic-resistance markers from therapeutic and vaccine plasmid DNA vectors. The presence of an antibiotic resistance gene in the plasmid backbone is considered undesirable by regulatory agencies, due to the potential transfer of antibiotic resistance to endogenous microbial flora and the potential activation and transcription of the genes from mammalian promoters after cellular incorporation into the genome.

Elimination of antibiotic resistance from the plasmid offers additional advantages, including elimination of potential metabolic stress caused by expression of the antibiotic marker gene product, and increased therapeutic potency if removal of the antibiotic marker results in a reduced plasmid size (e.g., the KanR gene is ~1 kb). Further, the use of antibiotics in fermentation culture requires expensive process validation of antibiotic removal during plasmid purification, to prevent contamination of the final product with residual antibiotics.

Nature Technology has designed an antibiotic-free selection system (*Figure 1*). Vectors with this selection system incorporate and express a 150 bp RNA-OUT antisense RNA. RNA-OUT represses expression of a counter-selectable marker (*SacB*) from the host chromosome. *SacB* encodes a levansucrase, which is toxic in the presence of sucrose. Plasmid selection is achieved in the presence of 0.5% sucrose.

Low Metabolic Burden Fermentation

Constitutive high plasmid copy number throughout the fermentation process is not necessary or desirable. Maintaining high copy numbers during biomass accumulation creates an environment in which plasmid-free cells have a significant growth advantage.

Plasmid-mediated metabolic burden can inhibit biomass growth and may lead to stability or quality problems (e.g., deletions or dimerization) with many plasmids. Thus, maintaining low cell stress/metabolic burden during biomass accumulation, and inducing high plasmid copy numbers for only the final portion of the process leads to higher volumetric plasmid yields while preserving plasmid quality.

This has been achieved by using a temperature-inducible, fed-batch process (*Figure 2A*) with an optimized semi-defined medium. The initial temperature setpoint is 30°C to keep the plasmid copy number at its minimum. Feed medium containing concentrated glycerol is added according to an exponential feeding strategy to control biomass growth at about 0.12 h⁻¹.

After sufficient biomass growth, the temperature is shifted to 42°C and growth continues for up to about one doubling of cell mass. This process has resulted in volumetric plasmid yields up to 2.2 g/L, and specific plasmid DNA yields up to 51 mg/g DCW (5% of the total dry cell weight). *Figure 2B* shows a time profile from a fermentation that reached a plasmid yield of 2.1 g/L.

Production of Unstable Plasmids

Based on our data, alternative high-yield plasmid production processes, which use 37°C continuously, are unlikely to perform well with many unstable or suboptimal plasmids due to increased metabolic burden. For example, pVLTrap, a 6.7 kb retroviral plasmid vector containing two long terminal repeats (LTRs, known to cause stability problems) was produced successfully in DH5 α at 785 mg/L using the aforementioned low metabolic burden fermentation process.

When grown under identical conditions, but at 37°C, the region between the LTRs had been entirely deleted by the end of the fermentation (*Figure 3*), and the plasmid yield only reached 214 mg/L. Thus, the use of the low metabolic burden process facilitates production of otherwise unstable plasmids in high-yielding strains, such as DH5 α , eliminating the need to use specialized stabilizing host strains.

Importantly, maintaining a low metabolic burden should begin as early as the transformation process. This is especially true with toxic and unstable plasmids. Numerous inserts can confer low-yield plasmid production on an otherwise high-yielding plasmid backbone. Propagation of cultures at 30°C, rather than 37°C, for manufacturing glycerol stocks can dramatically improve yields when producing plasmids containing toxic inserts.

This has been demonstrated by the following experiment: Three DNA Vaccine plasmids containing influenza hemagglutinin (HA) genes from H1, H3, or H5 serotypes were evaluated. Two of these genes, from H1, and H3 serotypes, are inserts known to lower plasmid yield. Fermentation yields from DH5 α glycerol stocks of these DNA vaccine plasmids, manufactured at either 30°C or at 37°C, are summarized in the table.

Glycerol stock viability was determined after >1 week at -80°C storage. Application of reduced-temperature glycerol stock production, coupled with the inducible fermentation process, solved the problem of manufacturing difficult plasmids, and resulted in successful production of 3/3 plasmids compared to only 1/3 using standard transformation at 37°C.

Nature Technology's temperature-inducible fermentation process has been successfully scaled up to 300 L and has been used for GMP production of DNA vaccine plasmids. Because of the ability to produce previously unstable and toxic plasmid DNA, as well as optimized plasmids at high yields, this low metabolic burden process is ideal as a generic plasmid DNA production platform.

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