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Abstract

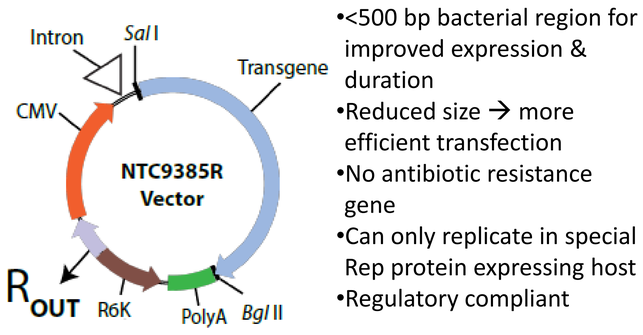
Plasmid based DNA vaccines are emerging as a promising alternative to traditional vaccines due to several advantages, including faster production of DNA plasmids using *E. coli*. However, further increases in transgene expression are needed to meet efficacy requirements for various non-viral gene therapy and DNA vaccination applications. While existing minicircle DNA technology has been shown to offer improved levels and durations of transgene expression by removal of the bacterial region from the plasmid, low manufacturing yields may be a barrier to widespread use of minicircle DNA for vaccination.

Nature Technology Corporation's (NTC's) minimalized Nanoplasmid™ vectors utilize RNA-OUT (R_{OUT}) antibiotic-free selection and replace the large 1000 bp pUC replication origin with a novel, 300 bp, R6K-derived mini-origin (**Fig. 1**). Reduction of the spacer region linking the 5' and 3' ends of the transgene expression cassette to <500 bp remarkably increases plasmid-mediated transgene expression. Host strains expressing heat-inducible, high copy R6K replication (Rep) proteins have been developed for selection and propagation of Nanoplasmid. This is an additional Nanoplasmid safety factor since mini-origin vectors can only replicate within the engineered Rep protein-expressing *E. coli* host strain (**Fig 2**).

With years of expertise in plasmid production, NTC and VGXI have successfully implemented NTC's HyperGRO™ fed-batch fermentation process for traditional plasmid and Nanoplasmid production at yields >1 g/L. However, production of plasmids containing large antigen-coding inserts may have various challenges. Bacterial cell machinery may not be able to produce high cell growth during fermentation due to a large gene insert in the plasmid. A modified HyperGRO process was developed by NTC to overcome this cell growth inhibition. Plasmid pNano1, a 6689 bp Nanoplasmid with a gene insert of 5018 bp, was successfully produced by VGXI using NTC's modified HyperGRO process, with high end cell density of OD_{600} 90.1 and volumetric yield of 0.696 g/L.

Further host strain engineering to repress plasmid copy number during biomass growth resulted in pNano1 volumetric yield of 2.4 g/L after 42°C induction of high copy Nanoplasmid amplification (**Fig. 8**), which is near the highest published fermentation yield for any plasmid.

Materials and methods



- <500 bp bacterial region for improved expression & duration
- Reduced size → more efficient transfection
- No antibiotic resistance gene
- Can only replicate in special Rep protein expressing host
- Regulatory compliant

Fig. 1: NTC9385R Nanoplasmid™ vector.

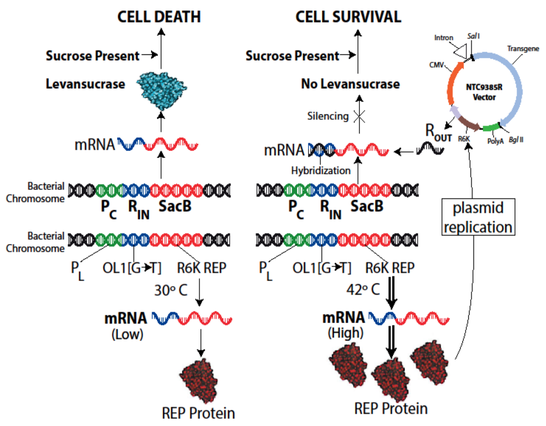


Fig. 2: Antibiotic-Free (AF) RNA-OUT-R6K plasmid selection and propagation.

Plasmids

- pNano1 (6689 bp), Nanoplasmid consisting of 1671 bp NTC9385R backbone (**Fig 1**) with 5018 bp gene insert.
- NTC8382-1 (8023 bp), pUC origin, antibiotic-free (RNA-OUT) backbone with the same large gene insert.

Plasmid and Nanoplasmid production host strains

- *E. coli* **NTC4862**: DH5α-derived with chromosomally integrated RNA-IN-*Sac*B (levansucrase) expression cassette for antibiotic-free, sucrose selectable pUC plasmids.
- *E. coli* **NTC821601**: DH5α-derived with chromosomally integrated RNA-IN-*Sac*B (levansucrase) and temperature inducible R6K Rep expression cassettes for antibiotic-free, sucrose selectable R6K miniorigin Nanoplasmids.
- *E. coli* **NTC1050811**: DH5α-derived with chromosomally integrated RNA-IN-*Sac*B (levansucrase) and temperature inducible R6K Rep expression cassettes for antibiotic-free, sucrose selectable R6K miniorigin Nanoplasmids, with an additional arabinose inducible Lambda cI repressor.

Fermentation

14L fermentors (New Brunswick Scientific) were employed at NTC and VGXI to perform 10L fermentations. A semi defined formulation was used for base and feed media, and 30→42°C temperature profiles were used to induce high copy plasmid amplification. Exponential feeding is used to control the specific growth rate (**Fig. 5**) during the early fed-batch phase for biomass accumulation. With pUC-type plasmids, the specific growth rate is controlled at $\mu = 0.12\ h^{-1}$. However, with Nanoplasmids containing a large insert, this low growth rate results in early growth inhibition due to excessive plasmid content. Controlling the specific growth rate at $\mu = 0.26\ h^{-1}$ (near 30°C μ_{max}) keeps the Nanoplasmid copy number low to allow high biomass accumulation, before the temperature induced high copy Nanoplasmid amplification.

Purification

Figure 3. Schematic of the VGXI manufacturing process from receipt of a client plasmid through cGMP delivery of filled vials.

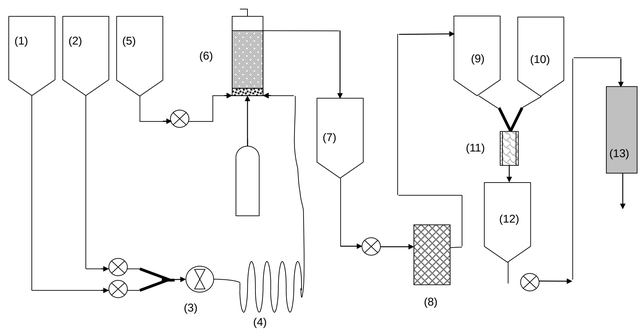


Figure 4. Schematic of the VGXI cell lysis process using AIRMIX[4] technology and a scalable continuous capture method. (1) Cell Resuspension, (2) Alkaline Lysis Solution, (3) Low Residence High Shear Static Mixer, (4) Lysis Hold Coil, (5) Neutralization Precipitation Solution, (6) AIRMIX Column, (7) Crude Lysate, (8) Lysate Filtration, (9) Filtered Lysate Holding Tank, (10) USP Purified Water, (11) Static Inline Mixer, (12) AEX Membrane Load, (13) AEX Capture Membrane.

The VGXI Patented Airmix® Lysis Process is one of the most critical steps in the purification process. During this step, the cell paste of pNano1 was suspended in the resuspension solution and mixed with the lysis solution using a static mixer. The introduction of air bubbles at the precipitation step aids in low shear mixing and results in more efficient removal of impurities like genomic DNA and host cell proteins. The lysis step was optimized for the plasmids with the NTC backbone by increasing the incubation time of the crude lysate before filtering. The plasmid concentration increases during this incubation, perhaps by diffusion of plasmid from the cell debris and better reannealing of the plasmid strands. As a result, higher step yield was observed at the end of lysis.

Results

NTC Process Development

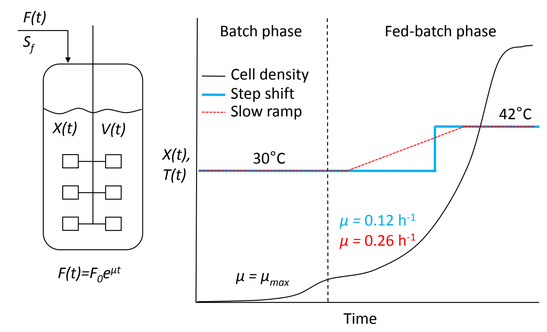


Figure 5. Illustration of NTC's HyperGRO inducible fed-batch fermentation process using an exponential feeding strategy and 30→42°C step or slow ramp temperature profile.

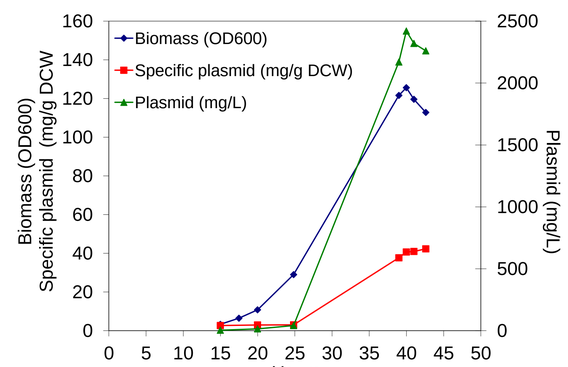


Fig. 6: NTC8382-1 (pUC origin) growth and yield profile of a standard HyperGRO fed-batch fermentation using 30→42°C step induction at ~55 OD_{600}. Host = NTC4862. Final plasmid yield was 2.2 g/L.

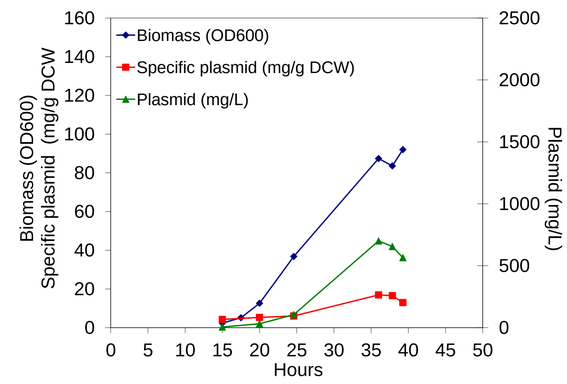


Fig. 7: pNano1 (NTC9385R Nanoplasmid) growth and yield profile of a modified HyperGRO fed-batch fermentation. Feeding for μ=0.26h^{-1}; 30→42°C slow ramp temperature induction. Host = **NTC821601.** Final Nanoplasmid yield was 0.57 g/L.

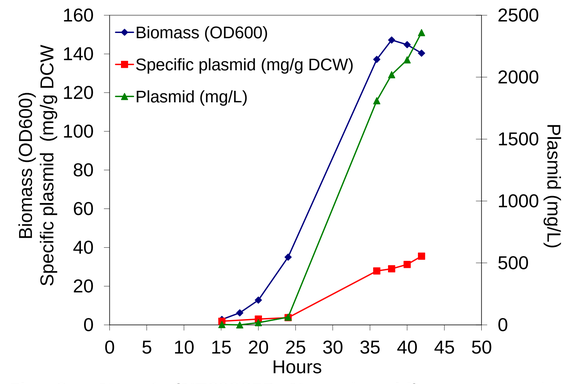


Fig. 8: pNano1 (NTC9385R Nanoplasmid) growth and yield profile of a modified HyperGRO fed-batch fermentation. Feeding for μ=0.26h^{-1}; 30→42°C slow ramp temperature induction. Host = **NTC1050811**. 0.02% arabinose used in base medium to repress growth phase copy number. **Final Nanoplasmid yield was 2.4 g/L**.

VGXI Production and Purification of pNano1 in *E. coli* NTC821601

The faster exponential feeding ($\mu = 0.26\ h^{-1}$ for Nanoplasmids was initiated at EFT (elapsed fermentation time) 15h accumulate biomass and keep the plasmid copy number low. At EFT 26h, a linear feeding rate was used with a slow ramp up of temperature from 30°C to 42°C to slowly induce plasmid production. At EFT 37.5h, a holding step at a lower temperature was performed for 30 minutes to allow replication completion of new plasmid molecules to the supercoiled form, increasing yield and quality.

A high volumetric plasmid yield of 0.696 g/L and specific plasmid yield of 2.77g/kg of cell paste, and final OD600 of 90.1 was achieved. At the end of the fermentation process, the cells were harvested using a semi-continuous centrifugation process.

pNano1 was purified from the cell paste as described (**Figs. 3 & 4**), achieving impurity levels (HCP, RNA, gDNA, endotoxin) much lower than FDA approved levels for clinical use (**Table 1**).

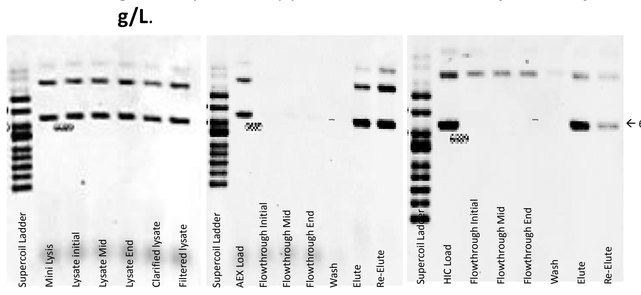


Fig. 9: Gel analysis of lysis, Anion Exchange and Hydrophobic Interaction Chromatography Process for pNano1 production in cell line NTC821601.

Assay	Units	pNano1
Concentration by A_{260}	mg/mL	9.5
Purity by $A_{260/280}$		2.0
Restriction		CTS
pH		7.3
Host Cell Genomic	%	0.3
Host Cell Protein (HCP)	%	≤0.02
Host Cell RNA	%	≤0.1
Endotoxin	EU/mg	0.5
Forms by HPLC		
Total Supercoil	%	94
Total Circular	%	99

Table 1: Quality Control Testing of Purified Nanoplasmid

Conclusions

NTC's Nanoplasmid™ is a next generation technology for plasmid production with a promising future. Nanoplasmid™ advantages include:

- Smaller spacer region → improved expression
  → improved immunogenicity
- No antibiotic resistance marker.
- Much higher production yields (>2 g/L) compared to minicircles.
- Safety: engineered production host required; Nanoplasmids cannot replicate in endogenous or environmental bacteria.
- Smaller overall size → higher potency
  → increased shear resistance during delivery
  → more efficient downstream purification

Fermentation process modifications overcame cell growth inhibition to allow high yield production of Nanoplasmids. Further host strain engineering resulted in Nanoplasmid production at similar or higher yields than pUC-origin plasmids (**Fig. 8** vs. **Fig 6**.).

VGXI has effectively implemented HyperGRO process modifications for Nanoplasmid production at the 10L scale. As a leader in the cGMP plasmid production, VGXI is committed to embolden innovation in the field to provide high quality plasmid DNA products.

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