RNA based plasmid selection system for antibiotic-free DNA vaccine vector production

Aaron E. Carnes B.Sc.Ch, Jeremy Luke, B.A., Justin Vincent, B.Sc., Clague Hodgson Ph.D., and James Williams Ph.D.

Nature Technology Corporation, 4701 Innovation Dr., Lincoln, NE, 68521 .

Abstract

Antibiotic resistance markers, typically kanamycin resistance (kanR), allow selective retention of plasmid DNA during bacterial fermentation and are the most commonly utilized selectable markers. However, to ensure safety, 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: 1) the potential transfer of antibiotic resistance to endogenous microbial flora; and 2) the potential activation and transcription of the genes from mammalian promoters after cellular incorporation into the genome. Here, we describe the development and application of a novel antibiotic-free (AF) selection system (**Fig. 1**). Vectors with this selection system (**Fig. 4**) incorporate and express a 150 bp RNA-OUT antisense RNA. RNA-OUT represses expression of a counter-selectable marker (*Sac*B) from the host chromosome. *Sac*B encodes a levansucrase, which is toxic in the presence of sucrose. Sucrose selectable DNA vaccine vectors combine antibiotic-free selection with highly productive fermentation manufacturing (>1 g/L plasmid DNA yields; **Table 1, 3**), while improving *in vivo* expression of encoded proteins (**Fig. 3**). The RNA-OUT selectable marker can be used to retrofit existing *kanR* DNA vaccine plasmids into antibiotic-free vectors. Interestingly, a minimum vector size for high yield production of small plasmids is reported. These vectors are safer, more potent, alternatives for DNA therapy or vaccination.

Materials and Methods

Strains and plasmids

E. coli **DH5α**: F- Φ 80d*lac*ZΔM15 Δ(*lac*ZYA -*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_K-, m_K+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1; **NTC4862** DH5α att_λ::$P_{5/6\ 6/6}$-RNA-IN- SacB, catR

Plasmids NTC8385, NTC8485 and NTC8685 contains a 150 bp DraIII-KpnI RNA-OUT based sucrose selectable marker. NTC8485 and NTC8685 contain the high yield SV40-PASBH backbone also present in the kanR vector NTC7485 (Williams *et al.,* 2009).

NTC fermentation media (Carnes *et al.,* 2006) was modified to contain 0.5% sucrose for plasmid selection. Fermentation was as described in Carnes *et al*., 2009.

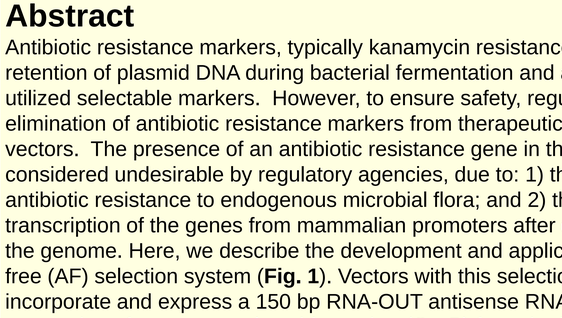


Fig. 1: Antibiotic Free (AF) selection

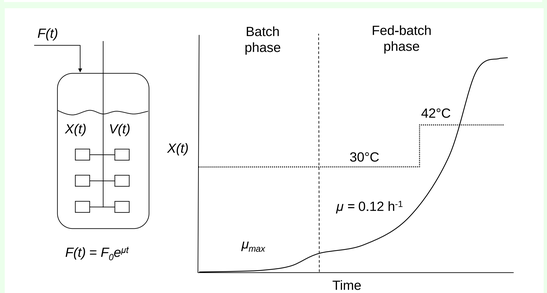


Fig. 2: Inducible fed-batch plasmid fermentation process

Results

Production/expression

Antibiotic-free (AF) selection vectors direct higher transgene expression than alternative vectors such as gWIZ or pVAX vectors (**Fig. 3**) and are compatible for high yield production (>1 g/L; **Tables 1 & 3**) with a low metabolic burden inducible (30-42ºC) fed-batch fermentation process (Carnes *et al.,* 2006; Williams *et al*., 2009) (**Fig. 2**).

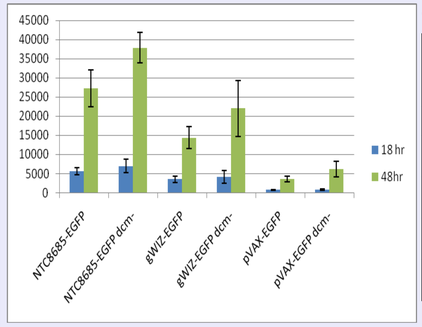


Fig. 3: AF vector expression

Table 1: AF vector Fermentation yields

Construct	Size (kb)	Selection	Ferm Yield (mg/L)	Relative Copy Number*
gWIZ-EGFP	5.75	Kan	1370	2.8
gWIZ-EGFP-AF2	4.89	Sucrose	1065	2.7
NTC8385-KGF	3.59	Sucrose	775	2.5
NTC8485-KGF	3.80	Sucrose	1210	4.4
NTC8685-KGF	3.68	Sucrose	1055	3.5

* Ratio of specific plasmid yield/size, (mg/L/OD_{600})/kb

gWIZ vector retrofit to AF selection

The gWiz vector (VR1012 equivalent), a commonly utilized *kanR* DNA vaccine vector, was retrofitted with the RNA-OUT selectable marker (both potential orientations) by precise replacement of the *kanR* gene (ATG-TAA) and fermentation yield and eukaryotic expression determined. RNA-OUT orientation 2 (**Fig. 4;** AF2) has equivalent fermentation copy number and eukaryotic expression as the parent vector (**Table 1).**

AF vector *in vivo* expression and immunogenicity

AF and KanR influenza H5 hemagglutinin (HA) DNA vaccine plasmids were tested for immunogenicity. Expression levels for each vector were determined, using SEAP expressing versions of the vectors. Either 3 μg HA or SEAP plasmid was injected intra-muscular (IM, quadriceps) of female BALB/c mice (6-8 weeks old) in a single dose (day 0) prior to electroporation (EP) using the MedPulser® system (Inovio Biomedical, San Diego CA). Serum samples were taken and tested for SEAP or anti-HA IgG response. The results are summarized in **Table 2** and demonstrate the antibiotic free NTC backbone (**Fig. 4**) is equivalent or superior to the *kanR* backbone for expression and immunogenicity (Luke *et al*., 2009).

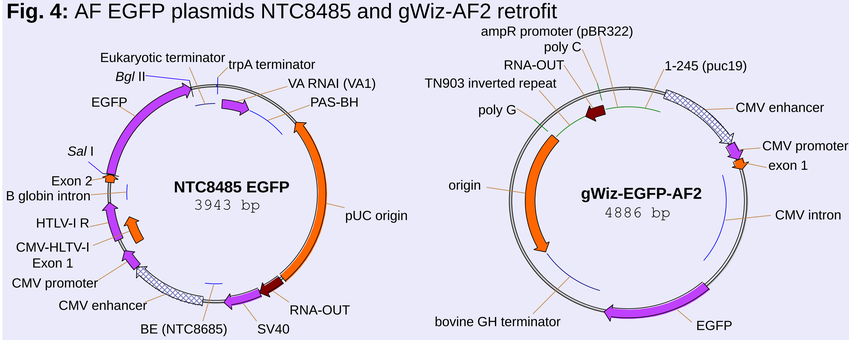
Table 2: KanR and AF vector *in vivo* expression (SEAP) and immunogenicity (HA)*.

Vector Selection	4 day SEAP (ng/ml)	7 day SEAP (ng/ml)	14 day SEAP (ng/ml)	21 day anti-HA2 IgG (total) (Abs 1/12,500)	21 day anti-HA2 IgG2a (Abs 1/500)	21 day anti-HA2 IgG1 (Abs 1/500)	% sero-conversion (1/2500)
(kan)	216 ±147	300 ±121	158 ±43	0.173 ±0.101	0.318 ±0.229	0.140 ±0.069	6/8
(Sucrose)	241 ±71	305 ±83	243 ±43	0.302 ±0.134	0.483 ±0.236	0.377 ±0.384	7/8
Control†				0.066 ±0.010	0.079 ±0.023	0.086 ±0.020	1/8

* Results presented as average ± standard deviation.

† All day 0 SEAP undetected. For HA, kanR-SEAP was the negative control plasmid.

Fig. 4: AF EGFP plasmids NTC8485 and gWiz-AF2 retrofit



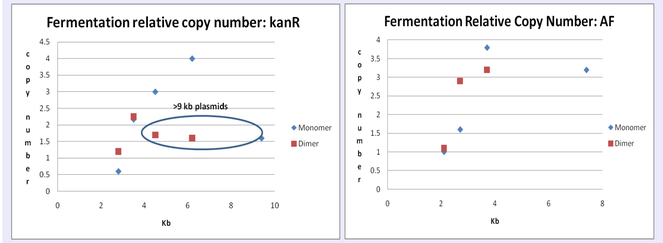


Fig. 5: Vectors < 3kb have reduced copy number

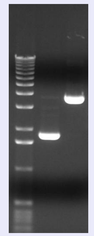


Fig. 6: AF Monomer and dimer production

Table 3: Vectors < 3kb have reduced copy number

Backbone/ Selection	Size (kb)	Ferm Yield (mg/L)	Relative copy #
gWIZ (Kan)	**2.8**	70	**0.4**
	5.7	1370	2.8
NTC7485 (Kan)	**2.8**	186	**0.6**
	5.7 (**Dimer**)	810	**1.2**
	2.9	320	**1.0**
	3.5	845	2.2
	6.9 (**Dimer**)	1290	2.2
	4.5	1440	3.0
	6.2	2200	4.0
NTC8485 (Sucrose)	**2.1**	193	**1.0**
	4.2 (**Dimer**)	420	**1.1**
	2.7	394	1.6
	5.4 (**Dimer**)	1150	2.9
	3.7	1220	3.8
	7.4 (**Dimer**)	1740	3.2
	8.3	1740	2.5

Small vector replication

KanR and AF vectors < 3kb have reduced copy number after plasmid induction in the fermentation process (**Fig. 5, Table 3**). Monomer and dimer fermentations demonstrated:
1) replication defect is due to reduced plasmid copy number induction;
2) Reduced plasmid induction correlates with monomer subunit size < 3kb; and
3) Inducible fermentation process produces high quality monomer and dimer DNA (**Fig. 6**).

Conclusions

We report the development of antibiotic-free (AF) DNA Vaccine vectors that combine >1 gm/L fermentation yields of high quality plasmid with improved *in vivo* expression and immunogenicity. The RNA based selectable marker has general utility for retrofitting antibiotic-containing vectors such as gWiz. The AF expression vector backbones are optimized to exceed a newly identified size threshold for high copy plasmid replication.

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