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Small-scale GMP production of plasmid DNA using a simplified and fully disposable production method

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ABSTRACT

In the past years, the demand for small batches of clinical grade plasmid DNA has been growing. For that purpose, we designed and qualified a scaled-down Good Manufacturing Practices (GMP) production method, able to produce small batches (1-4 mg) of plasmid. The developed method does not require any complex production equipment and utilizes only disposable production materials, which makes it easy to implement and simplifies line-clearance. We have successfully used this method to produce several small batches of two different plasmids. The produced plasmids, both formulated in an Electroporation Buffer, are mixed and filled into small, single-use, aliquots. Quality control confirmed the robustness of the developed method and a stability study showed that the final formulation is stable for at least two years. The final patient formulation will be subsequently used in a phase I/II clinical trial in which retina cells of patients with Age Related Macular Degeneration, are transfected. The presented production method can be generically used for other plasmid constructs and final formulation designs.

1. Introduction

Both gene therapy and (stem) cell therapy have become attractive treatment options in a wide variety of diseases over the past decades (Ginn et al., 2013; Gorabi et al., 2018; Melero et al., 2014). Besides the commonly used viral vectors, non-viral gene delivery methods using plasmid DNA are particularly interesting, since they are in general cheaper and faster to produce. For clinical applications, Good Manufacturing Practice (GMP) grade plasmid DNA is required. We have previously described a method to produce large scale (> 100 mg) batches of plasmid DNA that we used for DNA vaccination purposes at our institute (Quaak et al., 2008; Samuels et al., 2017). In the past years, the demand for small quantities of plasmid DNA for phase I/II trials has been growing. Besides for clinical phase I/II studies, small aliquots of plasmid can also be useful for animal experiments and pilot studies in which only small amounts are required. For this purpose, a small scale manufacturing process was developed. The production method is a scaled-down version of a previously described GMP production process (Quaak et al., 2008) and is able to deliver small quantities (1-4 mg) of plasmid DNA. This production method has two major advantages compared to the original larger-scale method: 1) it does not require any specialized production equipment, such as a bioreactor or chromatography system, 2) all materials are disposable, avoiding any cleaning validation, which saves additional time and costs. By using a small scale and disposable Tangential Flow Filtration (TFF) method to concentrate plasmid and exchange solvent, no organic solvents are required.

The small scale plasmid DNA production method was subsequently used for the production of two plasmids, pFAR4-CMV-SB100x-SV40 and pFAR4-ITRs-CMV-PEDF-BGH, which are miniplasmids devoid of an antibiotic resistance gene. A mixture of the two plasmids is being developed for the *ex vivo* electroporation of retina cells in a clinical trial. The two plasmids (pFAR4-PEDF and pFAR4-SB100x) are produced

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independently as two separate bulk plasmids, mixed in a 1:16 w/w ratio and aliquoted in 25 µl per glass vial as final, single-use, patient formulation under aseptic conditions. From this final formulation, a pilot and clinical batch were produced which confirmed reproducibility and stability of the produced product. The final drug product (investigational medicinal product) will be used in a the phase I study in patients with age related macular degeneration.

2. Materials and methods

2.1. General

The plasmid DNA was produced and formulated under aseptic conditions, in two grade B cleanrooms (Interflow, Wieringerwerf, The Netherlands). One cleanroom is dedicated to bacteria handling (upstream processing), the other is dedicated to plasmid purification and formulation of the final product (downstream processing). Both cleanrooms contain a grade A biosafety cabinet in which all critical proceedings were performed. Both cleanrooms are subjected to a monitoring program for viable and non-viable particles, both at operating as well as at resting state. The whole process was carried out according to Good Manufacturing Practice (GMP) guidelines (EudraLex (2019)). During the entire manufacturing and formulating process, only disposable products were used. Buffer components, pharmaceutical excipients and primary packaging materials used in the manufacture of plasmids were of European Pharmacopeia (Ph.Eur.) grade (if possible) and provided with a Certificate of Analysis (CoA) by the supplier. All materials and excipients were approved on the basis of in-house quality controls and vendor qualification. All buffers for bacterial lysis, pre clarification and purification were prepared by the production facility of the pharmacy department of the Antoni van Leeuwenhoek-The Netherlands Cancer Institute (AvL-NKI). The Electroporation Buffer for final formulation was supplied by 3 P Biopharmaceuticals (Navara, Spain), which was developed in the FP7 EU TargetAMD consortium. This iso-osmotic buffer is suited for *ex vivo* electroporation of retina cells and tested for sterility and endotoxins. The composition of the other buffers was kindly supplied by Qiagen and listed in Table 1.

2.2. Plasmid design

For this product, two plasmids were produced; pFAR4-ITRs-CMV-PEDF-BGH and pFAR4-CMV-SB100x-SV40 (Fig. 1) (Pastor et al., 2018; Thumann et al., 2017).

2.2.1. Description of pFAR4-ITRs-CMV-PEDF-BGH (pFAR4-PEDF) miniplasmid

The rationale behind gene therapy with SERPINF1 is that the protein that it encodes, Pigment epithelium-derived factor (PEDF), is important for the survival and function of the retina because of its neuroprotective and antiangiogenic capacities (Barnstable and Tombran-Tink, 2004; Subramanian et al., 2013). The pFAR4 gene vector backbone has a size of 1130 bp. The PEDF mRNA was isolated from retinal pigmented epithelium of a 19 year old male and retrotranscribed into cDNA (provided by S. Johnen, the University Hospital Aachen, Germany). The ITRs (inverted terminal repeats) and CMV PEDF BGH insert was cloned into the pFAR4 vector by Le Centre National de la Recherche Scientifique (CNRS). The primers for PCR amplification contain restriction sites and PCR products were enzymatically digested after amplification. Enzymatic digestions were cleaned up using Nucleospin Extract II Macherey Nagel kit and ligated over night at 16 °C using T4 DNA Ligase. Plasmid construct is depicted in Fig. 1A, and its features are summarized in Table 2. Construct identity was confirmed by full sequence analysis.

Table 1
Buffer compositions, pH and conductivity.

Buffer	Composition	pH
TE buffer	50 mM Tris, 10 mM EDTA	8.0
Lysis buffer	200 mM NaOH, 1% SDS	Not measured
Neutralisation buffer	3 M KAc	5.5
Endotoxin removal buffer	750 mM KCl, 50 mM KAc, 10% v/v Triton X-100	5.0
Equilibration buffer	750 mM KCl, 50 mM KAc	5.0 (conductivity 80 mS/cm)
Wash buffer	1.7 M KCl, 50 mM Tris-Cl	7.2
Elution buffer	2.0 M NaCl, 50 mM Tris	7.5
Electroporation buffer	Confidential, buffer is iso-osmotic	7.2

2.2.2. Description of pFAR4-CMV-SB100x-SV40 (pFAR4-SB100x) miniplasmid

As for pFAR4-PEDF, the pFAR4 vector backbone was used. Plasmid pCMV(CAT) SB100X was obtained from Z. Izsvak (Max Delbrück Center for Molecular Medicine, Berlin, Germany). The miniplasmid pFAR4-SB100x was constructed by means of an overnight enzymatic digestion using NdeI and SalI. The insert consists of a hyperactive Sleeping Beauty transposase (SB100x) that mediates stable genomic integration of genes flanked by ITR regions (see pFAR4-PEDF described above). Miniplasmid construct is depicted in Fig. 1B, and its features are summarized in Table 2. Construct identity was confirmed by full sequence analysis.

2.3. Upstream processing

2.3.1. Master Cell Bank (MCB) and Bacterial growth

For the production of pFAR antibiotic-free plasmids a mutant *Escherichia coli* strain, called TM#47-9a was used. The genotype of the strain is MG1655 thyA-ΔendA-ΔrecA. Without the plasmid, these bacteria require broth supplemented with thymidine for growth. After transformation, broth without supplemented thymidine can be used to apply selective pressure (Pastor et al., 2018). To make a batch of competent cells, the TM#47-9a strain was plated on a BBL™ Select APS™ LB Broth (APS) (BD Biosciences, Franklin Lakes, New Jersey, USA) agar plates supplemented with thymidine (30 µg/mL final concentration) (Sigma Aldrich, Saint Louis, Missouri, USA) and incubated at 37 °C. The second day, a colony from the plate was cultured in 5 mL APS broth supplemented with thymidine at 37 °C at 200 rpm. The optical density of the culture was measured at a wavelength of 600 nm (OD_{600}). When the OD_{600} was between 0.6 and 0.8, TM#47-9a cells were made competent with 0.1 M CaCl2. Glycerol was added and aliquots of 1 mL/cryo vial were “snap frozen” in 96% ethanol and dry-ice and stored at −80 °C.

To create the MCB of plasmid containing cells, competent cells were transformed with either of the two pFAR4 miniplasmids using a standard heat shock method (Russell, 1989). Transformed cells were plated on a sterile APS plate without thymidine. On these selective plates, only cells containing the pFAR4 miniplasmid will be able to grow. One single colony from the plate was isolated and grown in 175 mL APS Broth medium at 37 °C and 200 rpm. When the OD_{600} of the culture reached 0.6-0.8, 96 mL of glycerol 85% was added (30%v/v) and aliquots of 1 mL/cryo vial were “snap frozen” in 96% ethanol and dry-ice and stored at −80 °C.

For further plasmid production, one vial of the MCB was pre-cultured in 20 mL APS broth in a 50 mL tube at 37 °C and 200 rpm. After 6 hrs, all cells were transferred to two 1 L baffled Erlenmeyers containing 500 mL APS each and grown for approximately 18 hrs. OD_{600} was measured before harvesting for information.

2.4. Downstream processing

2.4.1. Lysis and pre-clarification

Bacteria were harvested by spinning down cell cultures in two

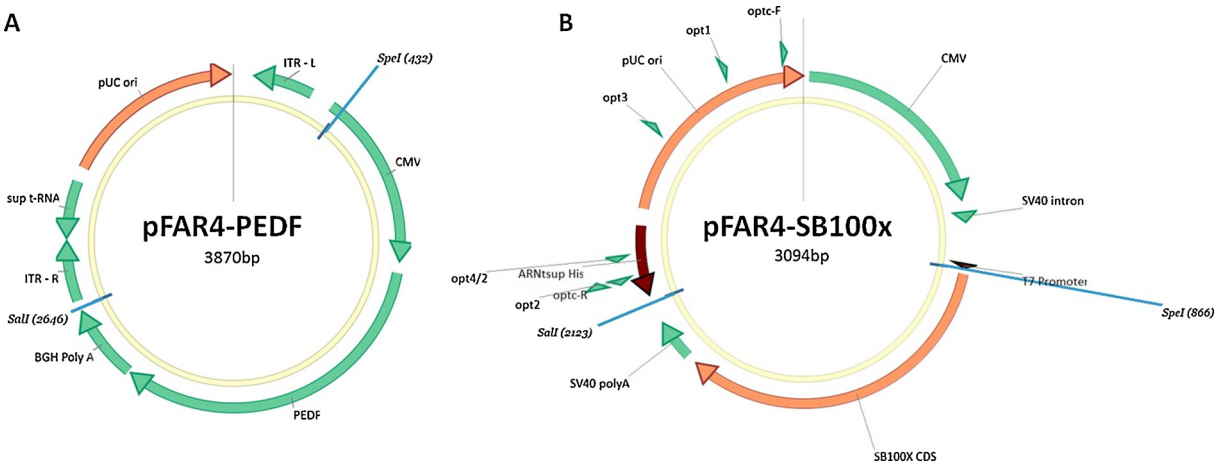

Fig. 1. Schematic representation of **A)** pFAR4-PEDF (3870 bp) and **B)** pFAR4-SB100x (3094 bp).

Table 2
pFAR4-SB100x and pFAR4-PEDF domains with specific features and functions.

Plasmid	Features	Description
pFAR4-SB100x	SV40 Intron	Increases translation
	SB100x gene	Sleeping Beauty 100x coding gene
	SV40 polyA	Efficient transcription termination and polyadenylation of mRNA
pFAR4-PEDF	PEDF gene	Pigment epithelium-derived factor coding gene
	BGH polyA	Efficient transcription termination and polyadenylation of mRNA
	ITR-R	Inverted terminal repeat sequence, allowing Transgene transposition
	ITR-L	Inverted terminal repeat sequence, allowing Transgene transposition
Shared	Origin of Replication	High-copy number replication
	CMV promoter	Permits efficient, high-level expression of the recombinant protein
	ARNtsup His	Suppressor t-RNA

Table 3
Overview of In Process Controls (IPCs).

IPC 1	Bacterial culture	1 mL
IPC 2	Lysate	1 mL
IPC 3	Flow-through after loading of lysate onto purification column	1 mL
IPC 4	Flow-through of equilibration step	1 mL
IPC 5	Flow-through of washing steps	1 mL
IPC 6	Concentrated fraction before Electroporation Buffer exchange	25 µL
IPC 7	Concentrated fraction after Electroporation Buffer exchange	50 µL
IPC 8	Final product (after sterile filtration)	300 µL

500 mL conical tubes (Corning, New York, USA) for 15 minutes at 4000*g*. Cells were washed twice with TE Buffer (see Table 1 for buffer composition) and resuspended in a final volume of 25 mL TE Buffer.

The use of animal derived enzymes is not preferred for the production of clinical grade plasmid DNA (Eon-Duval and Burke, 2004a; Eon-Duval et al., 2003; Ferreira et al., 1999). In the test runs prior to establishing the currently described manufacturing process, the necessity of additional RNase was assessed. In the absence of RNAse, a very low amount of DNA was recovered during the purification phase, because residual RNA was competing with plasmid DNA for the binding to the anion exchange resin. It was therefore decided to use bovine spongiform encephalopathy (BSE)-free certified RNase A.

A final concentration of 10 mg/L RNase (Sigma-Aldrich) was added to the concentrated culture in TE Buffer just before lysis. Lysis was performed at room temperature using 25 mL Lysis Buffer (see Table 1 for buffer composition). After 10 minutes, 25 mL pre-chilled (4 °C) Neutralization Buffer (Table 1) was added and lysed culture was filtered using a QIAFilter Mega-Giga Cartridge (Qiagen, Hilden, Germany) to obtain a clear solution. Subsequently, 1/10th volume of Endotoxin Removal Buffer (Table 1) was added to the lysate and conductivity was adjusted to ± 80 mS/cm using 3 M KCl as reference.

2.4.2. Packing of anion exchange purification column and purification

For each 165 mL of original culture, 1 disposable (Econo-Pac® Chromatography Columns, Biorad, Hercules, California, USA) column was packed with 2 grams Ultrapure 500 resin (Qiagen). The resin was suspended in 10 mL Elution Buffer (see Table 1 for buffer composition) and transferred to the column were it is allowed to settle down for 10 minutes before removing the stopper from the bottom of the column. The column volume was 2 gram * 2.6 = 5.2 mL. Each anion exchange column was primed with 3 column volumes of Equilibration Buffer (Table 1).

The cleared lysate was loaded onto the column and allowed to flow through. The column was then washed with 8 column volumes of Equilibration Buffer, and subsequently with 8 column volumes of Wash Buffer (Table 1). pDNA was harvested from the column using 10 mL of Elution Buffer, which was collected in a sterile tube.

2.4.3. Concentration and sterile filtration

A single use, Tangential Flow Filtration (TFF) system mPES MicroKros® Filter Modules P/N C04-E10-05-S (SpectrumLabs Europe, Breda, The Netherlands), with a pore size of 10 kD, was used to concentrate plasmid solution to approximately 1 mg/mL. For this, plasmid solution was pressured through the TFF membrane by hand, using sterile syringes. Subsequently, Elution Buffer was exchanged for Electroporation Buffer using the same TFF system in eight steps. After this exchange, plasmid solution was collected from the TFF system and sterile filtered through a Minisart® 0.2µM Filter (Sartorius, Goettingen, Germany). Filter integrity was controlled after sterile filtration. In process controls (IPCs) were taken during purification and concentration process (see Table 3) and used for quality control assays.

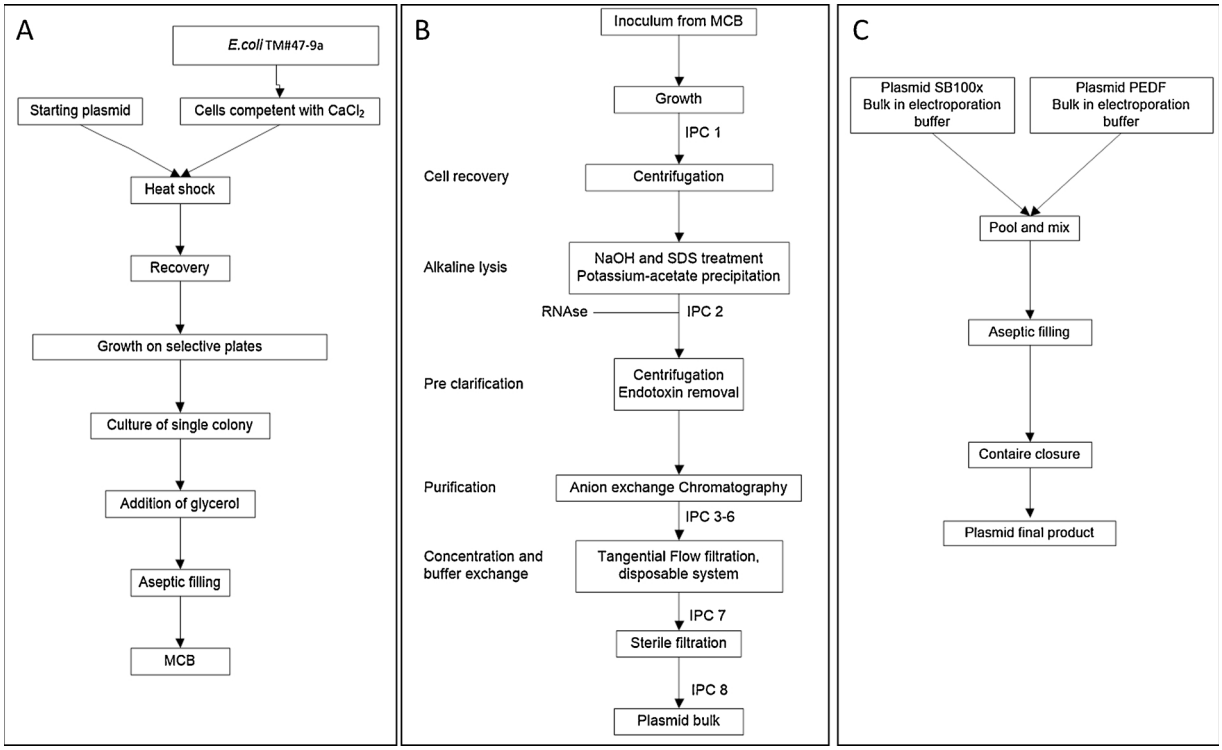

Fig. 2. Process flow sheets for the production of pFAR4 SB100x/PEDF: production of Master Cell Bank (MCB) (A), plasmid bulk drug (B) and pFAR4-CMV-SB100x-SV40/ pFAR4-ITRs-CMV-PEDF-BGH mixture sterile solution (C).

2.5. Formulation

To make up the final dosage form, the two plasmids were further diluted with the required Electroporation Buffer, mixed in a pFAR4-SB100x / pFAR4-PEDF in a 1:16 w/w ratio, which was optimized for this specific clinical application, and filled and finished in small, single-use vials. Aliquots of 25 µL were filled into glass snap ring vials with integrated Micro-Insert, 32 x 11.6 mm; 3850 ng pDNA/vial, and sterile capped with UltraClean Closure, 11 mm Aluminium/Silicone Caps (AGP, Bochum, Germany). Vials were stored at − 20 °C.

2.6. Quality control

All chemicals used for quality control were of analytical grade and used without further purification.

2.6.1. Agarose gel electrophoresis (AGE) including restriction endonuclease digestion

All IPCs as well as the final products were analyzed by gel electrophoresis, using self-cast 1% agarose gels and running at 30 V of 21 hours. After electrophoresis, gels were stained for 1.5 hours with a 1x Sybr green I solution (Sigma Aldrich) and subsequently DNA bands were visualized with UV light and a gel imaging device (GeneGenius, Westburg B.V., Leusden, The Netherlands). The image was recorded with a computer operated camera, and the intensity of the bands of interest were measured and compared against linear and circular DNA markers (New England Biolabs, Ipswich, Massachusetts, USA) loaded onto the same gel.

In order to also examine linear conformation of the final product, plasmid DNA was cleaved by restriction enzymes SpeI and SalI in buffer M (Invitrogen, Carlsbad, California, USA). Samples were diluted to a concentration of ~50 µg/mL. Subsequently 2 µL of 6x loading dye (New England Biolabs) was added to 10 µL of sample and directly loaded onto the gel. For the determination of residual *E. coli* host RNA/DNA, the sample was diluted to 50 ng/µL. Of the resulting solution, 10 µL was mixed with 2 µl of 6x loading dye and this was subsequently loaded onto the gel.

To semi-quantitative determine the ratio of the two pFAR plasmids in the final formulation, a standard curve of both plasmids was made with a range of 30 to 90 ng per slot. The final plasmid formulation was loaded on the same gel undiluted or diluted 1/20 v/v. Using the GeneGenius software, the areas under the curve for each plasmid was determined and the relative concentration of both plasmids in the mixture was calculated to determine the final ratio. This test was performed under GMP.

2.6.2. UV analysis

UV analysis was used to determine the concentration of purity of the plasmid DNA. Samples were diluted 4x to an approximate concentration of 40 µg/mL and measured with a Biophotometer (Eppendorf, Hamburg, Germany). Absorbance was measured at 230, 260, 280 and 320 nm. This test was performed under GMP.

2.6.3. Capillary gel electrophoresis (CGE) analysis

To determine purity in terms of pDNA topology (OC, CCC, linear), CGE was performed at the PlasmidFactory (Bielefeld, Germany).

2.6.4. Protein analysis

A bicinchoninic acid (BCA) assay from Thermo scientific Pierce (Waltham, Massachusetts, USA) was used to measure residual protein content. Bulk plasmid and the final drug product were exchanged in Electroporation Buffer. The proteins in the Electroporation Buffer were interfering with the test, making the samples unsuitable to use in this assay. It was therefor decided to analyze IPC 6 in the BCA assay, which corresponds to the concentrated fraction before buffer exchange (see Table 3). This test was performed under GMP.

2.6.5. Sequencing

To confirm plasmid integrity, both plasmids were sequenced (both strands) by Qiagen. Obtained sequences were compared with the

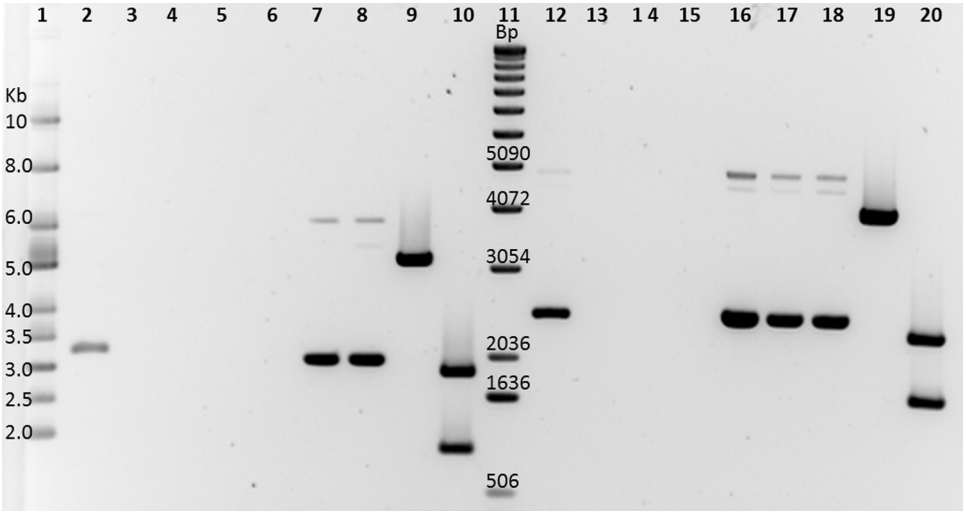

Fig. 3. Analysis of IPCs and bulk product pilot batch by 1% agarose gel electrophoresis.

Lane	Identity	IPC	Step in process	Expected band size
1	supercoiled DNA ladder			
2	SB100X	2	Lysate	3094 pb on supercoiled DNA ladder
3	SB100X	3	Flow-through after loading of lysate onto purification column	No bands expected
4	SB100X	4	Flow-through of equilibration step	No bands expected
5	SB100X	5	Flow-through of washing steps	No bands expected
6	SB100X	6	Concentrated fraction before Electroporation Buffer exchange	Not loaded
7	SB100X	7	Concentrated fraction after Electroporation Buffer exchange	3094 pb on supercoiled DNA ladder
8	SB100X	8	Final product (after sterile filtration). Unrestricted.	3094 pb on supercoiled DNA ladder
9	SB100X	8	Restriction SpeI	3094 bp on 1 kb plus DNA ladder
10	SB100X	8	Restriction SpeI and SalI	1257 bp and 1837 bp on 1 kb plus DNA ladder
11	1 kb plus DNA ladder			
12	PEDF	2	Lysate	3870 pb on supercoiled DNA ladder
13	PEDF	3	Flow-through after loading of lysate onto purification column	No bands expected
14	PEDF	4	Flow-through of equilibration step	No bands expected
15	PEDF	5	Flow-through of washing steps	No bands expected
16	PEDF	6	Concentrated fraction before Electroporation Buffer exchange	3870 pb on supercoiled DNA ladder
17	PEDF	7	Concentrated fraction after Electroporation Buffer exchange	3870 pb on supercoiled DNA ladder
18	PEDF	8	Final product (after sterile filtration). Unrestricted.	3870 pb on supercoiled DNA ladder
19	PEDF	8	Restriction SalI	3870 bp on 1 kb plus DNA ladder
20	PEDF	8	Restriction SpeI and SalI	2214 bp and 1656 bp on 1 kb plus DNA ladder

Table 4
Quality Control of the produced Master Cell Bank with Abbreviations: AGE, agarose gel electrophoresis.

Test item	Specification	Master Cell Bank
Appearance	Cream coloured, frozen cell suspension	Conform
Sequencing	Conform reference	Conform
AGE analysis: *Identification*	90-110% expected size	99.3%
AGE analysis: *Restriction analysis*	Conform expected size	Conform
Identification genus	E. Coli	Conform
Purity test (monosepsis)	ICH guidelines	No other microorganisms than host cell line present
Approved?	Complies to all tests	yes

theoretical sequence of the plasmids.

2.6.6. Bioburden and endotoxin

Bacterial endotoxins (using the gel clot LAL assay) and sterility tests were performed according to Ph.Eur. (chapter 2.6.1 and chapter 2.6.14). All tests were performed according to standardized analytical procedures using reference standards and equipment qualified for its purpose. This test was performed under GMP.

2.6.7. Identity and Purity of MCB

Identity of the MCB is determine by PCR of extracted DNA. The resulted sequence is machted with a validated database to find a match with a known micro organism.

Purity of the MCB was determined with a monosepsis assay according ICH guidelines Q5D. In this test, the MCB is grown on plates and inspected for aberrant colonies (at least a 1000 colonies are inspected

Both tests were performed by Sinesis Life Science, Leiden, The Netherlands under GMP regulations.

2.6.8. qPCR

Residual genomic E.Coli DNA was determined using a quantitave PCR method by Plasmid Factory (Bielefeld, Germany)

2.6.9. Uniformity of dosage forms

Uniformity of dosage forms was determined of filled final aliquots by weighing filled and empty dried vials, according to Ph. Eur. (chapter 2.9.40). This test was performed under GMP.

2.6.10. Stability upon storage

Stability of the 1:16 w/w pFAR4-SB100x/pFAR4-PEDF final formulation of the pilot and clinical batches, stored at −20 °C in the dark,

Table 5
In-process and quality control test results of all batches bulk drug manufactured. Abbreviations: OD600, optical density at 600 nm; TFF, tangential flow filtration; AGE, agarose gel electrophoresis; BCA, bicinchoninic acid; CGE, Capillary Gel Electrophoresis; UV, ultra violet. CCC, Covalently Closed Circular ; OC: Open circle.

Test item	Specification	Bulk product			
		pFAR4-CMV-SB100x SV40 pilot batch	pFAR4-CMV-PEDF-BGH pilot batch	pFAR4-CMV-SB100x SV40 clinical batch	pFAR4-CMV-PEDF-BGH clinical batch
In-process controls:					
OD_{600} after culture	For information	1.8	2.3	2.8	2.6
Growth times (hours)	For information	18	23	17	23
Amount DNA in eluate (mg)	For information	0.76 mg	4.0 mg	5.0 mg	4.1 mg
Recovery after TFF (%)	For information	69%	100%	75%	58%
Amount DNA final product after filtration (mg)	For information	0.38 mg	4.17 mg	3.0 mg	1.81 mg
Quality control:					
Appearance	Clear, colourless solution	Conform	Conform	Conform	Conform
Sequencing	Conform reference	Conform	Conform	Conform	Conform
UV analysis: *Concentration*	For info (mg/mL)	1.12	1.14	1.15	0.85
UV analysis: *Purity*	$A_{260/280}$ = 1.80 – 1.95	1.83	1.82	1.82	1.85
CGE analysis: Homogeneity (ccc-content)	≥ 85% CCC	94.0	95.6	94.0	95.3
AGE analysis: *Identification*	90-110% expected size	98.5%	106.1%	99.2%	99.0%
AGE analysis: *Restriction analysis*	Conform expected size	Conform	Conform	Conform	Conform
AGE analysis: *Purity*	Report size of other visible bands (compared to supercoiled DNA (ladder)	OC form Dimer supercoiled	OC form Dimer supercoiled	OC form Dimer supercoiled	OC form Dimer supercoiled
Residual *E.coli* host DNA	< 5% (< 0.05 mg/mg plasmid)	Conform	Conform	Conform	Conform
Residual *E.coli* host RNA	≤ 4% (≤ 0.04 mg/mg plasmid)	Conform	Conform	Conform	Conform
BCA assay	≤ 5 µg/mL protein	Conform	Conform	Conform	Conform
Sterility	sterile	Conform	Conform	Conform	Conform
Bacterial endotoxins	< 10EU/mg	Conform	Conform	Conform	Conform
Approved?	Complies to all tests	yes	yes	yes	yes

Table 6
Overview of expected sizes and actual values of restricted plasmid DNA products. Plasmid and fragment sizes approximate the predicted sizes and all fall within the specifications (90-110% of expected size).

		Expected fragment size	Determined fragment size	Aberration
PEDF	Single restriction	3870 bp	4106 bp	6,1%
	Double restriction	2214 bp	2229 bp	0,7%
		1656 bp	1623 bp	−2,0%
SB100x	Single restriction	3094 bp	3059 bp	−1,1%
	Double restriction	1257 bp	1217 bp	−3,2%
		1837 bp	1795 bp	6,1%

was evaluated in triplicate at several time points up to 24 months. Vials were visually inspected. Content was measured by UV analysis (see above for details). Supercoiled DNA content was assessed by CGE (PlasmidFactory).

3. RESULTS

3.1. Production process

The production process contains three major steps: production of a Master Cell Bank, bulk plasmid DNA preparation and final clinical formulation. An overview of the plasmid production process is shown in Fig. 2. Two bulk production runs were performed for each plasmid: a pilot run and a run resulting in the clinical product, which makes a total of four individual bulk productions. In process controls of those four runs are summarized in Table 3. Quality control analysis of the MCB and bulk miniplasmids pFAR4-PEDF and pFAR4-SB100x and control samples taken during the production processes, were all conformed to specifications (see Fig. 3, Table 4 and Table 5), demonstrating that the used production method is robust. An overview of expected sizes and actual values of restricted plasmid DNA products is given in Table 6.

Table 7
Quality control on final formulation (mixture pFAR4- SB100x / pFAR4-PEDF in a 1:16 ratio) of pilot batch and clinical batch. Abbreviations: CCC = Covalently Closed Circular (= supercoiled), AGE = Agarose Gel Electrophoresis, CGE: Capillary Gel Electrophoresis.

Test item	Specification	Final formulation	
		pFAR4-SB100x / pFAR4-PEDF pilot batch	pFAR4-SB100x / pFAR4-PEDF clinical batch
Quality control:			
Appearance	Clear, colourless solution	Conform	Conform
UV analysis: *Concentration*	90-110%of labelled content	108%	94%
UV analysis: *Purity*	$A_{260/280}$ = 1.80 – 1.95	1.83	1.83
CGE analysis: Homogeneity (ccc-content)	≥ 85% CCC	94.7%	87.7%
AGE analysis: *Identification*	Size bands similar size as bulk	Conform	Conform
AGE analysis: *Ratio*	1:12 – 1:20	1:15.5	1:19
Residual gDNA (by qPCR)	For information only	4.4%	5.5%
Uniformity of Dosage units	Weight variation of 10 vials ≤15%	9%	13%
Sterility	Sterile	Conform	Conform
Bacterial endotoxins	< 100 EU/mg	< 10EU/mg	< 10EU/mg
Approved?	Complies with all tests	Yes	Yes

The yield of the first pilot batch pFAR4-CMV-SB100x SV40 was relatively low (378 µg, see Table 5). This was due to the relatively low cell density reached at the end of the bacterial growth. By prolonging the growth time of the pre-culture to 6 hrs during the subsequent miniplasmid productions, a higher yield was obtained. Since the required amount of SB100x plasmid was relatively low, the amount of

Table 8
Results of a stability study of pFAR4-SB100x/pFAR4-PEDF final formulations for the pilot and clinical batches at -20 °C in the dark, Abbreviations:; UV, ultra violet; CGE: capillary gel electrophoresis, ccc: covalently closed circular - supercoiled plasmid topology for the highest expression efficacy.; c: clear.

Test item	Specification	Final patient formulations									
		pFAR4-CMV-SB100/ pFAR4-PEDF (Pilot Batch)						pFAR4-CMV-SB100/ pFAR4-PEDF (Clinical Batch)			
	Time point (months)	QC	4	10	16	24	30	QC	7	15	21
Appearance	Clear, colourless solution	C	C	C	C	C	C	C	C	C	C
UV analysis											
Concentration	139-169 ng/µl	166	159	151	167	165	169	145	144	148	149
Purity	$A_{260/280}$ = 1.80 – 1.95	1.83	1.85	1.87	1.81	1.83	1.84	1.83	1.83	1.83	1.82
CGE analysis											
Homogeneity (ccc-content)	≥ 85% CCC	94.7	93.7	94.7	94.2	94.3	94.7	87.7	94.0	94.9	93.7
Complies y/n		y	y	y	y	y	y	y	y	y	y

plasmid produced was sufficient to cover the needs for the phase I/II study. The recovery after TFF varied between 58 and 100% between batches, which is acceptable for this application.

3.2. Clinical formulation development

Since this clinical trial requires *ex vivo* transfection of retina cells in the shortest timeframe possible to prevent cell death, a pre-mixture of both miniplasmids was requested as final drug product. Therefore, both pilot batches and clinical batches of pFAR4-PEDF and pFAR4-SB100x were diluted with Electroporation Buffer to the desired concentration, mixed together and filled & finished in small, single-use, glass vials at a final content of 3850 ng of total plasmid. Three hundred vials of the pilot and clinical batch of final patient formulation were filled and quality controlled (see Table 7). Both final formulations were conform specifications.

3.3. Stability upon storage

Stability of the final clinical formulation (both pilot batch and clinical products), was evaluated at multiple time points up to 2.5 years storage at −20 °C. Table 8 shows the shelf life results obtained thus far. Content and purity of all samples at each time point, still comply to all specifications (content 90-110% of labeled content and purity ≥85% supercoiled topology).

4. Discussion

The production method is a scaled-down version of a previously described process. Compared to our original proces by Quaak et al, this process is utilizing RNAse after lysing. We have observed during product development that residual RNA binds strongly to the used resin material (data not shown), thereby making the orginal process fairly inefficient. The use of animal derived RNA is not optimal, but acceptable for this application after a careful risk analysis. The product is BSE-free certified and the theoretical residual amount of RNAse in the final product is negligible low because of extensive media exchange steps. In the future, it would be recommendable to re-design this method in such way that it does not require the use of additional RNAse. This could be done by the addition of an extra precipitation step or by careful selection of resin and operating conditions during chromatography (Eon-Duval and Burke G., 2004b).

Compared to the large-scale process, no specialized production equipment, such as pumps, fermentation system and preparative chromatography system is required. In addition, all materials are fully disposable, lacking the need of comprehensive line-clearance or cleaning validation. This makes this process easy to apply.

The recovery of the plasmid after TFF was varying between 58 and 100%. Although this appears rather variable, it is an acceptable range for this process, since only a small amount of plasmid is required for this clinical application. Because the process handles small volumes, the relative loss and dead-volume in the TFF system is high, resulting in the somewhat variable recovery.

5. Conclusion

We have described a fully disposable, small scale plasmid DNA production method. The pDNA yield and purity using this strategy are comparable to methods that have been described before (Gorabi et al., 2018) and are sufficient for most phase I/II applications

The final drug product presented here consisted of a mixture of pFAR4-SB100x and pFAR4-PEDF miniplasmids in a 1:16 w/w ratio and complied to all specifications, exhibiting a high purity measured by both UV and AGE analyses and consistently high homogeneity in supercoiled plasmid topology (≥87.7%, see Table 6).

The final formulation has shown to be stable for at least 2.5 years at −20 °C; more data is currently collected in the stability program that continues to date. All executed runs were successful and the final drug product of each run complied to all quality requirements, vouching reproducibility of this GMP-compliant manufacturing process. This manufacturing method can be generically applied for the small scale production of other plasmid DNA constructs and will be easy to implement by other facilities.

Declarations of interest

The authors have no conflicts of interest to declare.

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