TABLE OF CONTENTS

LIST OF TABLES

3.2.S.2.3. CONTROL OF MATERIALS – SOURCE, HISTORY AND GENERATION OF PLASMIDS [AGC]

This section was originally prepared for plasmids used in the manufacture of Original drug substance. Presentations using Original drug substance have been discontinued but the manufacturing process information is continued to be used for plasmids used as starting materials for variant drug substances.

3.2.S.2.3.1. Plasmid Cell Bank and Linear DNA Template Manufacturer(s)

The plasmid master cell bank (MBC) manufacture and associated testing is performed in the Pfizer facility at 875 Chesterfield Parkway West, Chesterfield MO 63017.

The plasmid master cell bank inventory is stored in Pfizer's storage facilities located at 875 Chesterfield Parkway West, Chesterfield MO 63017 and 1 Burtt Road, Andover MA 01810. The master cell bank testing was performed at 4.2 1 ind. 4.2 1 ind. The master cell bank sequence testing was performed 4.2 1 ind.

The plasmid working cell bank (WCB) manufacture, starting material (linear DNA template) manufacture and associated testing are performed at AGC Biologics GmbH, Czernyring 22, 69115 Heidelberg, Germany. The plasmid WCB inventory is stored in AGC's GMP storage facilities located at AGC Biologics GmbH, Germany. The cell bank testing was performed at AGC Biologics GmbH and 4.2 1st ind The cell bank sequence testing was performed 4.2 1 ind.

3.2.S.2.3.2. Plasmid Cell Banking System, Characterization and Testing

Plasmid cell banks have been prepared in accordance with the following guidelines:
ICH Q5D *Derivation and characterization of cell substrates used for production of biotechnological/biological products*

Cell banking operations were performed in a controlled manufacturing area with appropriate precautions against adventitious contamination and cross-contamination from other cell lines. AGC used the MCB established by Pfizer Chesterfield. Details can be found in Section 3.2.S.2.3 Control of Materials – Source, History, and Generation of Plasmids [Pfizer Chesterfield].

3.2.S.2.3.2.1. Preparation of pST4-1525 Working Cell Bank

Manufacture of the WCB is done according to batch documentation. A pre-culture flask containing 4.2 1 ind. medium with 4.2 1 ind. of the master cell bank material is cultivated at 37°C and 180 rpm until OD_{600nm} 2-4 is reached. Cells are then harvested by adding cryopreservation media and filling of the cell suspension into labelled vials for the WCB. After cryopreservation of the filled vials using a controlled rate freezer the vials are finally transferred to storage in vapor phase of liquid nitrogen at ≤ -130 °C.

3.2.S.2.3.2.2. Release Testing of the Plasmid Working Cell Bank

The analytical methods at AGC for characterization, release testing and retesting of the Working Cell Bank (WCB) include tests for purity and quality (culture purity, contaminating phages, cell viability, retention of expression plasmid, plasmid copy number), identity of host strain, expression plasmid and expression gene (confirmation of *E. coli* species, genetic identity of *E. coli* strain, restriction digest of expression plasmid, sequence confirmation of gene and flanking regions).

Culture purity and identity testing performed on the plasmid WCB C-211901 provide confirmation that the cell bank is free from microbial and bacteriophage contamination and is of an *E. coli* lineage. The studies were designed in accordance with ICH Q5D guidelines.

Table 3.2.S.2.3-1 lists the release tests performed, acceptance criteria, and results for the WCB C-211901.

Table 3.2.S.2.3-1. Release Testing of Plasmid WCB Batch C-211901

Test	Method	Acceptance Criteria	Result
Purity			
Culture purity	Ph. Eur. 2.6.12 and 2.6.13	4.2 1 ind.	
Bacteriophage – lytic & lysogenic	Infection with T4 bacteriophage with/without irradiation	Free of bacteriophage and lysogenic prophage	Complies
Identity			
Host cell identity (Genotypic testing)	API 20 E patter by test kit	Identified as derived from *Escherichia coli*	4.2 1 ind.
Genetic identity of E. coli	RAPD fingerprint analysis of genomic DNA using PAGE/AGE	RAPD fingerprint corresponds to reference RAPD fingerprint.	RAPD fingerprint corresponds to reference RAPD fingerprint
Quality			
Cell viability	Plating on TSA	4.2 1 ind.	
Plasmid retention	Replica plating on LB agar with and without antibiotic.	4.2 1 ind. cells resistant to kanamycin	4.2 1 ind. cells resistant to kanamycin
Identity of expression construct and heterologous gene			
Restriction map analysis	Digestion with restriction enzymes and separation of DNA fragments on agarose.	Banding pattern on agarose gel corresponds to banding pattern of reference plasmid	Banding pattern on agarose gel corresponds to banding pattern of reference plasmid
DNA sequencing	DNA sequencing	DNA of the expression cassette corresponds to theoretical sequence	Complies

Abbreviations: WCB = working cell bank; Ph. Eur. = European Pharmacopoeia; CFU = colony forming unit; RAPD = randomly amplified polymorphic DNA; PAGE = polyacrylamide gel electrophoresis; AGE = agarose gel electrophoresis; TSA = tryptic soy agar; LB = lysogeny broth; RP = result pending

3.2.S.2.3.2.3. Preparation, Qualification and Storage of Renewal Plasmid Working Cell Banks (WCBs)

At this stage no renewal of plasmid working cell bank is defined.

3.2.S.2.3.2.4. Plasmid Cell Bank Stability Testing

To ensure the quality (shelf-life) of the existing WCB, a re-examination on cell viability, retention of plasmid and DNA sequence confirmation shall be performed every 5 years. If fermentation is carried out routinely, the quality of the WCB may be evaluated by process data generated during production to justify the stability and to ensure the shelf-life of WCB.

3.2.S.2.3.3. Linear DNA Template Manufacturing

Cells from the WCB are thawed and the culture is expanded in shake flasks, which are then used to inoculate the pre-fermenter. An aliquot of the pre-fermenter is used to inoculate the main culture in a 4.2 1 ind. fermenter and the fermentation step is ended by time. The entire fermentation process at AGC is performed without addition of antibiotics. After fermentation the cells are broken by chemical lysis. After filtration, the remaining solution containing plasmid DNA is concentrated, the buffer exchanged and then passed to Chromatography. During DSP, the DNA is purified by two sequential column steps (anion-exchange chromatography and hydrophobic interaction chromatography) and then linearized 4.2 1 ind. Finally, the linearization enzyme is removed by a third column (mixed-mode chromatography) 4.2 1 ind. The linear DNA template is aliquoted, filled and frozen.

Table 3.2.S.2.3-2. Process Flow for Linear DNA Template

Process Step	Process Description
Pre-culture I	One vial from the working cell bank is thawed and used to inoculate a shake flask (pre-culture I)..
Pre-culture II	One flask of pre-culture I is inoculated in the pre-fermenter (pre-culture II).
Main culture	The pre-culture II is transferred into a 4.2 1 ind. fermenter (total volume) and the fermentation is ended by time.
Cell harvest	The production fermenter is harvested by disc separator.
Cell lysis and RNA precipitation	Resuspension of cell mass; chemical cell lysis and pDNA preparation using static mixer and subsequent calcium chloride precipitation.
Ultrafiltration/ Diafiltration 1	Ultra- and diafiltration using hollow fiber modules to concentrate and buffer exchange in preparation for next chromatography step.
Anion Exchange Chromatography	4.2 1 ind. column w 4.2 1 ind. is used for capturing of pDNA material.
Hydrophobic Interaction Chromatography	4.2 1 ind. olumn is used for this polishing process step of pDNA material.
Ultrafiltration/ Diafiltration 2	Ultra- and diafiltration using hollow fiber modules to concentrate and buffer exchange followed by a 0.2 µm filtration.
Linearization	Linearization using wave rockers and single use bags.
Mixed-Mode Chromatography	A mixed-mode column with 4.2 1 ind. esin is used for this purification process step of the linearized DNA template.
Ultrafiltration/ Diafiltration 3	Ultra- and diafiltration using hollow fiber modules to concentrate and buffer exchange with 4.2 1 ind. for final fill.
Filtration and filling	The linear DNA template filtered via 0.2 µm filtration and stored frozen at ≤ -60 °C.

Purified water manufactured at the facility is used throughout the linear DNA template process and meets compendial requirements. A list of the raw materials used in the manufacture of the linear DNA template is provided in Table 3.2.S.2.3-3. All the materials used in the manufacture of the linear DNA template are animal origin free or the risk of carry-over of any adventitious contaminant from the animal derived material is very low risk to insignificant. Section 3.2.A.2 Adventitious Agents Safety Evaluation [BNT Marburg] is providing the evaluation of adventitious agent risk. All materials are sourced from approved suppliers. Inspection of materials received and examination of vendor certificate of analysis are performed for raw materials.

Table 3.2.S.2.3-3. Raw Materials and External Buffers Used in the Manufacture of Linear DNA Template

Raw Material	Supplier Grade[a,b]
1,4-Dithiothreitol (DTT)	Non compendial
Acetic acid	Ph. Eur., JP, USP
Ammonia solution 25%	Ph. Eur.
Ammonium sulfate	Ph. Eur., NF
Boric acid	Ph. Eur., NF
Calcium chloride dihydrate	Ph. Eur., JP, USP
Citric acid monohydrate	Ph. Eur., JP, USP
Cobalt sulfate heptahydrate	Non compendial
di-Sodium hydrogen phosphate dihydrate	Ph. Eur., USP
D(+)-Glucose monohydrate	Ph. Eur., USP
4.2 1 ind.	Non compendial
EDTA	Ph. Eur., ChP, JP, USP
Ethanol 96%	Ph. Eur.
HEPES	Non compendial
Hydrochloric acid 25%	Ph. Helv.
Iron(III)-chloride-hexahydrate	Ph. Eur., JPE
Magnesium acetate tetrahydrate	Ph. Eur.
Manganese(II) sulfate monohydrate	Ph. Eur., USP
85-% phosphoric acid	Ph. Eur., JPE, NF
Polypropylene glycol 2000	Non compendial
Potassium acetate	Ph. Eur.
Potassium chloride	Ph. Eur., USP, JP
Sodium chloride	Ph. Eur., JP, USP
Sodium dihydrogen phosphate monohydrate	BP, USP
Sodium dodecyl sulfate	NF
Sodium hydroxide	Ph. Eur.
Sodium molybdate dihydrate	Ph. Eur.
Tris(hydroxymethyl)amino methane	Ph. Eur., USP, ChP, JPC
Tris(hydroxymethyl)amino methane hydrochloride	Non compendial
Tris(hydroxymethyl)amino methane acetate	Non compendial
Zinc sulfate heptahydrate	Ph. Eur., USP

a. Equivalent can be used. Supplier Grades of incoming materials listed.
b. Gross visual, CoA check and ID test.

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT = DL-dithiothreitol; EDTA = edetate disodium dihydrate or ethylenediaminetetraacetic acid; NF = National Formulary; USP = United States Pharmacopeia; JP= Japan Pharmacopeia; Ph. Eur. = European Pharmacopeia; ChP = Chinese Pharmacopoeia; Ph. Helv. = Swiss Pharmacopoeia; JPE = Japanese Pharmacopoeia Excipients; JPC = Japanese Pharmaceutical Codex; BP = British Pharmacopoeia

The chromatography resins and filters used in linear DNA template manufacture are described in Table 3.2.S.2.3-4.

Table 3.2.S.2.3-4. Chromatography Resins and Filters Used in Linear DNA Template Manufacture

4.2 1 ind. [redacted] esin
4.2 1 ind. [redacted] resin
4.2 1 ind. [redacted] resin
30 kDa MWCO ultrafiltration membrane
Depth filters (deep bed filtration)
0.2 µm filters

3.2.S.2.3.4. Linear DNA Template Specifications

The release specifications for the linear DNA template are given in Table 3.2.S.2.3-5. The analytical control strategy includes sampling and testing of a selected number of attributes prior to linearization and the remainder of the attributes on the final linear DNA template. Those attributes analyzed prior to linearization are listed first (circular plasmid DNA) and those analyzed after linearization are listed second (linear DNA template).

Table 3.2.S.2.3-5. Linear DNA Template Specifications

Analytical Procedure	Quality Attribute	Acceptance Criteria
CIRCULAR PLASMID DNA		
Characteristics		
UV260	DNA Concentration	4.2 1 ind.
Restriction map	Poly A tail integrity	Bands at approximately 4.2 1 ind.
Identity		
Sanger sequencing	Identity of transcribed region	Homology to reference[a]
	Identity of poly A tail	Report results
Purity		
AEX-HPLC	Plasmid topology	Supercoiled form: 4.2 1 ind. Linear form: 4.2 1 ind.
Process-Related Impurities		
Assay RNA analysis	Residual host cell RNA	4.2 1 ind.
qPCR DNA analysis	Assay RNA analysis	
LINEAR DNA TEMPLATE		
Characteristics		
Appearance (clarity)	Clarity	Clear to slightly opalescent (not more turbid than reference suspension II)
Appearance (coloration)	Coloration	Colorless to slightly colored (not more colored than reference solution 4.2 1 ind.)
pH	pH	4.2 1 ind.
UV260	DNA Concentration	
Purity		
AEX-HPLC	Linearization Efficiency (Plasmid topology)	Linear form: 4.2 1 ind.
Process-Related Impurities		
Total residual protein by µBCA	Residual protein	4.2 1 ind.
Safety		
Bioburden	Bioburden	4.2 1 ind.
Endotoxin	Endotoxin	

a. Defined as 100% identity with DNA reference sequence in transcribed region excluding poly(dA:dT)-tract
Abbreviations: AEX-HPLC = anion exchange high pressure liquid chromatography; CFU = colony forming units; EU = endotoxin unit

3.2.S.2.3.5. Linear DNA Template Method Descriptions

Specific analytical procedures, including compendial and non-compendial methods, were used to assess characteristics, identity, purity and safety of the circular plasmid DNA and linear DNA template. Descriptions of the analytical procedures are provided below

3.2.S.2.3.5.1. Appearance (Clarity and Coloration)

The linear DNA template is assessed for clarity and coloration in accordance with the current European Pharmacopoeia procedure, Ph. Eur. 2.2.1 and Ph. Eur. 2.2.2, respectively.

3.2.S.2.3.5.2. pH

The linear DNA template is analyzed for pH in accordance with the current European Pharmacopoeia procedure, Ph. Eur. 2.2.3.

3.2.S.2.3.5.3. DNA Concentration by Spectroscopy

Using a spectrophotometer, the absorbance at 260 nm is used to determine the concentration of the circular plasmid DNA and linear DNA template. The specific absorption coefficient or absorptivity (a_{260}) for dsDNA (given below) is used for the concentration calculation.

a_{260} = 4.2 1 ind.

3.2.S.2.3.5.4. Identity by Restriction Mapping*

***Identification by Restriction Mapping was removed for future circular plasmid DNA batch analysis. The method summary is retained for historical purposes.**
A restriction endonuclease mapping method is used to determine identity of the circular plasmid DNA. The reference material and test samples are subjected to digestion using the following enzymes or groups of enzymes: 4.2 1 ind. all of which cleave at specific sites. The enzymatic digest produces a set of nucleic acid fragments which are separated through sieving via agarose gel electrophoresis and visualized by addition of a detection reagent. The bands of test samples are compared to the expected band pattern to confirm identity of the circular plasmid DNA by ensuring that all appropriate bands are present.

3.2.S.2.3.5.5. Identity of the Transgene Region by DNA Sanger Sequencing

Using Sanger sequencing the identity of the transgene region of circular plasmid DNA is determined. By involving electrophoresis and incorporation of chain-termination dideoxynucleotides by DNA polymerase the output sequence is compared to the nucleotide reference sequence.

3.2.S.2.3.5.6. Poly A Tail Integrity by Restriction Digestion

A restriction endonuclease mapping method is used to determine poly A tail integrity of the circular plasmid DNA. The test samples are subjected to digestion using the following enzymes or groups of enzymes: 4.2 1 ind. all of which cleave at specific sites. The enzymatic digest produces a set of nucleic acid fragments which are separated through sieving via agarose gel electrophoresis and visualized by addition of a detection reagent. The bands of test samples are compared to the theoretical band size values at approximately 4.2 1 ind. and approximately 4.2 1 ind. using the DNA ladder as a size reference to confirm integrity of the poly A tail by ensuring that all appropriate bands are present.

3.2.S.2.3.5.7. Plasmid Topology and Linearization Efficiency by AEX-HPLC

Anion-exchange chromatography is used to separate negatively charged nucleic acids and determine plasmid topology for circular plasmid DNA and linearization efficiency for linear DNA template. Chromatograms are compared to the respective reference material and amount of supercoiled and linear form evaluated by integration of peak area.

3.2.S.2.3.5.8. Total Residual Protein by µBCA

Quantification of protein impurities in linear DNA template using a protein assay kit. Residual protein in samples reduce Cu^{2+} to Cu^{+} ions, which form a purple-colored reaction product by chelation with bicinchoninic acid (BCA). Absorption of measured samples at 562 nm.

3.2.S.2.3.5.9. Host Cell DNA by qPCR

This assay for determination of host cell DNA of the circular plasmid DNA is based on the principle of the quantitative PCR. DNA is first isolated and purified. During the purification step, samples are digested by proteinase K, lysed and washed. The qPCR assay contains altogether 4.2 1 ind. A reporter dye is used as a fluorescent probe (4.2 1 ind.). The emitted fluorescence is proportional to the initial amount of target DNA amplified by 40 cycles of denaturation and elongation. The absolute quantification of the residual DNA in the sample is performed using the standard curve generated by the DNA Standard.

3.2.S.2.3.5.10. Host Cell RNA by Assay Kit

Before host cell RNA analysis of the circular plasmid DNA, the samples is treated with 4.2 1 ind. to prevent possible interference of DNA with the assay reagent. Following DNA digestion, residual RNA is analyzed with a selective fluorophore. RNA concentration is calculated based on a linear regression of a RNA standard curve (4.2 1 ind. 4.2 1 ind.

3.2.S.2.3.5.11. Residual Kanamycin by HPLC-FLD and ELISA

High performance liquid chromatography (HPLC) with fluorescence detection (FLD) is used as analytical procedure for detection residual kanamycin in circular plasmid DNA. The test method is a limit test based on a standard addition.
Wherever the samples matrix is not allowing for a determination of residual kanamycin by HPLC, an Enzyme-linked Immunosorbent Assay (ELISA) is used.

3.2.S.2.3.5.12. Bacterial Endotoxin Assay (Limulus Amebocyte Lysate [LAL])

The linear DNA template is assessed for bacterial endotoxin in accordance with the current European Pharmacopoeia procedure, Ph. Eur. 2.6.14, Method D.

3.2.S.2.3.5.13. Bioburden

The linear DNA template is tested for microbiological contamination in accordance with the current European Pharmacopoeia procedure, Ph. Eur. 2.6.12.

3.2.S.2.3.6. Linear DNA Template Method Verification/Qualification Data

The plasmid-specific methods are listed in Table 3.2.S.2.3-6. The compendial methods were verified following applicable Pharmacopoeias. The non-compendial methods for linear DNA template were qualified/verified to ensure analytical methods are sound and suitable for their intended use. A gap analysis will be performed and documented to identify any supplemental qualification to align with ICH requirements. The gaps identified will be addressed either prior to transferring the methods to relevant sites or during the transfer activities. The qualification/verification results are summarized in Table 3.2.S.2.3-6.

Table 3.2.S.2.3-6. Product Specific Qualification or Verification Summaries

<table>
<tr><th>Attribute</th><th>Method</th><th>Qualification-Verification Summaries</th></tr>
<tr><td colspan="3">CIRCULAR PLASMID DNA</td></tr>
<tr><td>DNA Concentration[a]</td><td>UV 260</td><td>
<table>
<tr><th>Parameter</th><th>Acceptance criteria</th><th>Result</th></tr>
<tr><td>System suitability</td><td>RSD of standard curve and controls : 4.2 1 ind.
4.2 1 ind.
4.2 1 ind.</td><td>RSD = 4.2 1 ind.</td></tr>
<tr><td>Specificity</td><td colspan="2">4.2 1 ind.</td></tr>
<tr><td>Linearity</td><td>Visual inspection of the plot demonstrates linearity</td><td>Visual inspection of the plot demonstrates linearity</td></tr>
<tr><td>Accuracy</td><td colspan="2" rowspan="3">4.2 1 ind.</td></tr>
<tr><td>Precision/ Repeatability</td></tr>
<tr><td>Precision/ Intermediate Precision</td></tr>
<tr><td>Range</td><td>The range is defined as the concentration interval for which all target criteria for linearity, accuracy and precision are fulfilled</td><td>4.2 1 ind.</td></tr>
<tr><td colspan="3">a. The investigation identified that the concentration of the in-process buffers used was too high. The method was amended and diluted buffers will be used.
b. Result of measurement with diluted buffers.</td></tr>
</table>
</td></tr>
<tr><td>Plasmid Topology[a]</td><td>AEX-HPLC</td><td>
<table>
<tr><th>Parameter</th><th>Acceptance criteria</th><th>Result</th></tr>
<tr><td>Specificity</td><td>The chromatogram of the formulation buffer injection does not contain any interfering peaks.</td><td>The chromatogram of the formulation buffer injection does not contain any interfering peaks.</td></tr>
</table>
</td></tr>
</table>

Table 3.2.S.2.3-6. Product Specific Qualification or Verification Summaries

Attribute	Method	Qualification-Verification Summaries
		The main peak is visually separated from product related species or degradation products, if visible. / The main peak is visually separated from product related species and degradation products for stressed material.
		Linearity: Visual inspection of the plot demonstrates linearity / Visual inspection of the plot demonstrates linearity
		Accuracy / Precision/ Repeatability / Precision/ Intermediate Precision: 4.2 1 ind.
		Range: The range is defined as concentration for which all target criteria for linearity, accuracy and precision are fulfilled / 4.2 1 ind.
		Limit of quantification: Lowest concentration which passes the following criteria: 4.2 1 ind. / 4.2 1 ind.
Residual host cell RNA	Assay RNA analysis	Repeatability: 4.2 1 ind. Specificity: Positive controls show positive result, negative controls show negative result Limit of quantification: 4.2 1 ind.
Residual host cell DNA	qPCR DNA analysis	Repeatability: 4.2 1 ind. Accuracy: 4.2 1 ind. Specificity: 1. Positive controls show positive result, negative controls show negative result 2. Absence of signals above LOQ in product matrix Limit of quantification: 4.2 1 ind.
LINEAR DNA TEMPLATE		
DNA Concentration[a]	UV 260	**Parameter** / **Acceptance criteria** / **Result** System suitability / RSD of standard curve and controls 4.2 1 ind. / 4.2 1 ind.

Table 3.2.S.2.3-6. Product Specific Qualification or Verification Summaries

Attribute	Method	Qualification-Verification Summaries		
		Specificity	Mean A_{260} of formulation buffer measurements	4.2 1 ind.
		Linearity	Visual inspection of the plot demonstrates linearity	Visual inspection of the plot demonstrates linearity
		Accuracy	4.2 1 ind.	
		Precision/ Repeatability		
		Precision/ Intermediate Precision		
		Range	The range is defined as the concentration interval for which all target criteria for linearity, accuracy and precision are fulfilled.	4.2 1 ind.
Plasmid Topology[a]	AEX-HPLC	**Parameter**	**Acceptance criteria**	**Result**
		Specificity	The chromatogram of the formulation buffer injection does not contain any interfering peaks.	The chromatogram of the formulation buffer injection does not contain any interfering peaks.
			The main peak is visually separated from product related species or degradation products, if visible.	The main peak is visually separated from product related species and degradation products for stressed material.
		Linearity	Visual inspection of the plot demonstrates linearity	Visual inspection of the plot demonstrates linearity
		Accuracy	4.2 1 ind.	
		Precision/ Repeatability		
		Precision/ Intermediate Precision		
		Range	The range is defined as concentration for which all target criteria for linearity,	4.2 1 ind.

Table 3.2.S.2.3-6. Product Specific Qualification or Verification Summaries

Attribute	Method	Qualification-Verification Summaries
		accuracy and precision are fulfilled
		Limit of quantification: Lowest concentration which passes the following criteria: 4.2 1 ind. — 4.2 1 ind.
Total Residual Protein[a]	µBCA Assay	**Parameter / Acceptance criteria / Result**
		System suitability: 4.2 1 ind. — RSD of standard curve and controls 4.2 1 ind. — 4.2 1 ind.
		Mean of calculated negative control 4.2 1 ind. — 4.2 1 ind.
		Recovery of positive control 4.2 1 ind.
		Specificity: Mean A_{562} of diluted formulation buffer is below mean A_{562} of 4.2 1 ind. BSA standard — A_{562} formulation buffer: 4.2 1 ind. A_{562} Standard: 4.2 1 ind.
		Linearity: Visual inspection of the plot demonstrates linearity — Visual inspection of the plot demonstrates linearity
		Accuracy / Precision/ Repeatability: 4.2 1 ind.
		Limit of quantitation: The range is defined as that interval of BSA concentration for which all target criteria for linearity, accuracy and precision are fulfilled — All acceptance criteria are fulfilled for spiked BSA concentrations between 4.2 1 ind.

Table 3.2.S.2.3-6. Product Specific Qualification or Verification Summaries

<table>
<tr><th>Attribute</th><th>Method</th><th>Qualification-Verification Summaries</th></tr>
<tr><td>Endotoxin[c]</td><td>USP <85>; Ph Eur. 2.6.14 & JP 4.01</td><td>Compendial qualification performed per local compendia.
The criteria of the standard curve was found to be valid (i.e. correlation coefficient (r) must be ≥ 0.980
The sample solution must not interfere with the test (e.g. inhibition/enhancement)
The sample must have a maximum valid dilution (MVD) established

Summary of Inhibition/Enhancement Data
<table>
<tr><th>Endotoxin Limit</th><th>λ (EU/mL)</th><th>Calculated MVD</th><th>Qualified Dilution</th></tr>
<tr><td>4.2 1 ind.</td><td>0.005 EU/mL</td><td>1:4000</td><td>1:50</td></tr>
</table>
Inhibition/Enhancement Results from the first two manufactured batches
<table>
<tr><td>Sample Dilution</td><td>Spike Recovery (%)</td><td>Results (EU/mL)</td></tr>
<tr><td>Batch 71703-212002: 1:50</td><td>127 %</td><td>< 0.250 EU/mL</td></tr>
<tr><td>Batch 71703-211901: 1:50</td><td>143 %</td><td>< 0.250 EU/mL</td></tr>
</table></td></tr>
<tr><td>Bioburden[c]</td><td>Ph. Eur. 2.6.12</td><td>Challenge Recovery Testing (based on Compendial guidance to ensure test articles are non-inhibitory to the recovery of inoculated organisms):
<table>
<tr><th>Organism</th><th>%Recovery</th></tr>
<tr><td>Pseudomonas aeruginosa</td><td>94 %</td></tr>
<tr><td>Bacillus subtilis</td><td>115 %</td></tr>
<tr><td>Staphylococcus aureus</td><td>116 %</td></tr>
<tr><td>Candida albicans</td><td>96 %</td></tr>
<tr><td>Aspergillus brasiliensis</td><td>98 %</td></tr>
</table></td></tr>
</table>

a. Platform Method Verification
b. Compendial Verified
c. Compendial Qualified

Abbreviations: AEX-HPLC = anion exchange high pressure liquid chromatography; BCA = bicinchoninic acid; USP = United States Pharmacopoeia; Ph. Eur. = European Pharmacopoeia; RSD = relative standard deviation; R^2 = regression coefficient; OP = operator; MVD = maximum valid dilution; EU = endotoxin unit; LOQ = limit of quantification

3.2.S.2.3.7. Linear DNA Template Batch Analysis

The batch analysis data for representative commercial scale batches of the linear DNA template are given in Table 3.2.S.2.3-7 below. At the time of manufacture of these batches, for some parameters different acceptance criteria compared to the commercial specifications (Table 3.2.S.2.3-5) applied. These differences are footnoted in the table below.

Table 3.2.S.2.3-7. Circular and Linear DNA Template Batch Analysis for Commercial Scale Batches

Analytical Procedure	Acceptance Criteria	Results for Batch			
		71703-211901	71703-212002	71703-214504	71703-214605
CIRCULAR PLASMID DNA					
Characteristics					
DNA Concentration (UV260)	4.2 1 ind.				
Identity					
Restriction map by agarose gel[d]	Comparable to reference[d]	Complies	Complies	Complies	Complies
Sanger sequencing - identity of the transcribed region	100% homology to reference	Complies	Complies	Complies	Complies
Sanger sequencing - identity of the poly A tail	Report results	Complies[c]	Complies[c]	Complies[c]	Complies[c]
Poly A tail integrity by agarose gel	Comparable to reference	Complies	Complies	Complies	Complies
Purity					
Plasmid topology by AEX-HPLC	Supercoiled form: 4.2 1 ind. Linear form 4.2 1 ind.	4.2 1 ind.			
Process Related Impurities					
Residual Host Cell RNA	4.2 1 ind.				
Residual Host Cell DNA					
LINEAR DNA TEMPLATE					
Characteristics					
Appearance (clarity)	Clear to slightly opalescent (not more turbid than reference suspension II)	Complies	Complies	Complies	Complies
Appearance (coloration)	Colorless to slightly colored (not more colored than reference solution 4.2 1 ind.	Complies	Complies	Complies	Complies
pH	4.2 1 ind.				
DNA Concentration (UV260)					
Purity					
AEX-HPLC	Linear form: 4.2 1 ind.				
Process Related Impurities					
Total residual protein	4.2 1 ind.				
Safety					
Bioburden					
Endotoxin					

Table 3.2.S.2.3-7. Circular and Linear DNA Template Batch Analysis for Commercial Scale Batches

Analytical Procedure	Acceptance Criteria	Results for Batch			
		71703-211901	71703-212002	71703-214504	71703-214605

a. Defined as 100% identity with DNA reference sequence in transcribed region excluding poly(dA:dT)-tract
b. Denotes specification at time of release of the batch. Specification was subsequently updated as shown in Table 3.2.S.2.3-5.
c. Accurate number of A (poly A stretch) upstream from sequence pattern GCATATGACT could not be verified.
d. Identification by Restriction Mapping was removed for future circular plasmid DNA batch analysis. Results are retained for historical purposes .

Abbreviations: AEX-HPLC = Anion exchange high pressure liquid chromatography; CFU = colony forming units; EU = endotoxin units; ND = not determined; RP = result pending

3.2.S.2.3.8. Circular and Linear DNA Template Standards

AGC used the circular plasmid DNA and linear DNA template standard materials provided by Pfizer (see Section 3.2.S.2.3 Control of Materials – Source, History, and Generation of Plasmids [Pfizer Chesterfield]) for the analysis of the circular and linear DNA template batches described above as well as for the characterization of their own standard materials. AGC prepared a circular plasmid DNA Control Standard (Contr.Std.-ES-71703-211901) and linear plasmid DNA Reference Standard (Ref.-Std.-71703-211901) from the first commercial batch 71703-211901 for future use. The test results for the standards are provided in Table 3.2.S.2.3-8 and Table 3.2.S.2.3-9. Two hundred 2.0 mL cryogenic vials were filled with 1000 µL for each standard. Storage at ≤ -60°C and retesting of the standard is done every year. The parent batch is also part of the stability program.

Table 3.2.S.2.3-8. Circular Plasmid DNA Reference Material Batch Contr.Std.-ES-71703-211901

Test	Method	Result
DNA Concentration	UV spectroscopy at 260 nm	4.2 1 ind.
Supercoiled form	AEX-HPLC	
Residual host cell DNA	Assay RNA analysis	

Abbreviations: AEX-HPLC = Anion exchange high pressure liquid chromatography

Table 3.2.S.2.3-9. Linear DNA Template Reference Material Batch Ref.-Std.-71703-211901

Test	Method	Result
DNA Concentration	UV spectroscopy at 260 nm	4.2 1 ind.
Linearization efficiency	AEX-HPLC	

Abbreviations: AEX-HPLC = Anion exchange high pressure liquid chromatography

3.2.S.2.3.9. Linear and Circular DNA Template Stability and Period of Use

3.2.S.2.3.9.1. Stability Plan

The shelf life of 18 months is established for both the circular plasmid DNA and the linear DNA template as described in Section 3.2.S.2.3 Control of Materials - Source, History and Generation of Plasmids [Pfizer Chesterfield].

The stability of circular plasmid DNA and linear DNA template is monitored using two representative batches at each stage (71703-211901 and 71703-212002). The stability samples are stored in representative 5 mL and 30 mL downscale versions of the 1000 mL PETG bottles used for long-term storage of linear DNA template, with a filling volume of 5 mL or 20 mL per stability container, respectively.

The stability programs have been established according to the protocols detailed in Table 3.2.S.2.3-10 through Table 3.2.S.2.3-13. In addition to DNA Concentration and Topology, Appearance (Clarity and Coloration) and pH of linear DNA template will be monitored during the stability study.

Table 3.2.S.2.3-10. Stability Protocol for Circular Plasmid DNA Stored at ≤ -60 °C (Long Term Storage Condition)

Analytical Procedure	Test Intervals (Months) [a]
UV (DNA concentration)	0, 4, 6, 12, 18, 24, 36
AEX HPLC (Topology)	0, 4, 6, 12, 18, 24, 36
Bioburden	0
Endotoxin	0

a. Initial data (t0) are from release testing.

Table 3.2.S.2.3-11. Stability Protocol for Circular Plasmid DNA Stored at 5 ± 3°C (Accelerated Storage Condition)

Analytical Procedure	Test Intervals (Months)[a]
UV (DNA concentration)	0, 4, 6
AEX HPLC (Topology)	0, 4, 6

a. Initial data (t0) are from release testing.

Table 3.2.S.2.3-12. Stability Protocol for Linear DNA Template Stored at ≤ -60 °C (Long Term Storage Condition)

Analytical Procedure	Test Intervals (Months) [a]
Appearance (Clarity)	0, 4, 6, 12, 18, 24, 36
Appearance (Coloration)	0, 4, 6, 12, 18, 24, 36
pH	0, 4, 6, 12, 18, 24, 36
UV (DNA concentration)	0, 4, 6, 12, 18, 24, 36
AEX HPLC (Topology) (Topology)	0, 4, 6, 12, 18, 24, 36

a. Initial data (t0) are from release testing.

Table 3.2.S.2.3-13. Stability Protocol for Linear DNA Template Stored at 5 ± 3 °C (Accelerated Storage Condition)

Analytical Procedure	Test Intervals (Months) [a]
Appearance (Clarity)	0, 4, 6
Appearance (Coloration)	0, 4, 6
pH	0, 4, 6
UV (DNA concentration)	0, 4, 6
AEX HPLC (Topology)	0, 4, 6

a. Initial data (t0) are from release testing.

3.2.S.2.3.9.2. Stability Data

The results of the stability studies for the circular plasmid DNA batches 71703-211901 and 71703-212002 stored at ≤ -60 °C and 5 ± 3 °C are provided in Table 3.2.S.2.3-14 through Table 3.2.S.2.3-17.

Table 3.2.S.2.3-14. Circular Plasmid DNA Batch 71703-211901 - Stability Data at ≤ -60°C Storage Condition

Assay	Acceptance Criteria	Time points					
		0	4M	6M	12M	18M	24M
DNA Concentration	4.2 1 ind.	4.2 1 ind.					S
Plasmid topology	Supercoiled form: 4.2 1 ind. Linear form: 4.2 1 ind.						S

Abbreviations: M = months; S = scheduled for testing

Table 3.2.S.2.3-15. Circular Plasmid DNA Batch 71703-211901 - Stability Data at 5 ± 3 °C Storage Condition

Assay	Acceptance Criteria	Time points		
		0	4M	6M
DNA Concentration	4.2 1 ind.	4.2 1 ind.		
Plasmid topology	Supercoiled form: 4.2 1 ind. Linear form: 4.2 1 ind.			

Abbreviations: M = months; S = scheduled for testing

Table 3.2.S.2.3-16. Circular Plasmid DNA Batch 71703-212002 - Stability Data at ≤ -60°C Storage Condition

Assay	Acceptance Criteria	Time points					
		0	4M	6M	12M	18M	24M
DNA Concentration	4.2 1 ind.	4.2 1 ind.					
Plasmid topology	Supercoiled form: ≥ 80.0 % Linear form: ≤ 5.0 %						

Abbreviations: M = months; S = scheduled for testing

Table 3.2.S.2.3-17. Circular Plasmid DNA Batch 71703-212002 - Stability Data at 5 ± 3 °C Storage Condition

Assay	Acceptance Criteria	Time points		
		0	4M	6M
DNA Concentration	4.2 1 ind.	4.2 1 ind.		
Plasmid topology	Supercoiled form: 4.2 1 ind. Linear form 4.2 1 ind.			

Abbreviations: M = months; S = scheduled for testing

The results of the stability testing for the linear DNA template batches 71703-211901 and 71703-212002 stored at ≤ -60 °C and 5 ± 3 °C are provided in Table 3.2.S.2.3-18 through Table 3.2.S.2.3-21.

Table 3.2.S.2.3-18. Linear DNA Template Batch 71703-211901 - Stability Data at ≤ -60°C Storage Condition

Assay	Acceptance Criteria	Time points					
		0	4M	6M	12M	18M	24M
Clarity	Clear to slightly opalescent (not more turbid than reference suspension II)	Complies	Complies	Complies	Complies	Complies	S
Coloration	Colorless to slightly colored (not more colored than reference solution 4.2 1 ind.	Complies	Complies	Complies	Complies	Complies	S
pH	4.2 1 ind.	4.2 1 ind.					S
DNA Concentration							S
Linearization Efficiency (Plasmid topology)	Linear form: 4.2 1 ind.						S

Abbreviations: M = months; S = scheduled for testing

Table 3.2.S.2.3-19. Linear DNA Template Batch 71703-211901 - Stability Data at 5 ± 3 °C Storage Condition

Assay	Acceptance Criteria	Time points		
		0	4M	6M
Clarity	Clear to slightly opalescent (not more turbid than reference suspension II)	Complies	Complies	Complies
Coloration	Colorless to slightly colored (not more colored than reference solution [redacted])	Complies	Complies	Complies
pH	[redacted]	[redacted]		
DNA Concentration				
Linearization Efficiency (Plasmid topology)	Linear form: [redacted]			

Abbreviations: M = months; S = scheduled for testing

Table 3.2.S.2.3-20. Linear DNA Template Batch 71703-212002 - Stability Data at ≤ -60 °C Storage Condition

Assay	Acceptance Criteria	Time points					
		0	4M	6M	12M	18M	24M
Clarity	Clear to slightly opalescent (not more turbid than reference suspension II)	Complies	Complies	Complies	Complies	Complies	S
Coloration	Colorless to slightly colored (not more colored than reference solution [redacted])	Complies	Complies	Complies	Complies	Complies	S
pH	[redacted]	[redacted]					S
DNA Concentration							S
Linearization Efficiency (Plasmid topology)	Linear form: [redacted]						S

Abbreviations: M = months; S = scheduled for testing

Table 3.2.S.2.3-21. Linear DNA Template Batch 71703-212002 - Stability Data at 5 ± 3 °C Storage Condition

Assay	Acceptance Criteria	Time points		
		0	4M	6M
Clarity	Clear to slightly opalescent (not more turbid than reference suspension II)	Complies	Complies	Complies
Coloration	Colorless to slightly colored (not more colored than reference solution [redacted]	Complies	Complies	Complies
pH	[redacted]			
DNA Concentration				
Linearization Efficiency (Plasmid topology)	Linear form: [redacted]			

[redacted]

Abbreviations: M = months; S = scheduled for testing