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BIOPHORUM RAW MATERIALS: CELL AND GENE THERAPY CRITICAL STARTING MATERIAL

FURTHER DISCUSSION ON PLASMIDS TO ESTABLISH RELEASE SPECIFICATIONS USING A RISK-BASED APPROACH TO MANAGE SUPPLY

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About BioPhorum

BioPhorum's mission is to create environments where the global biopharmaceutical industry can collaborate and accelerate its rate of progress, for the benefit of all.

Since its inception in 2004, BioPhorum has become the open and trusted environment where senior leaders of the biopharmaceutical industry come together to openly share and discuss the emerging trends and challenges facing their industry.

Growing from an end-user group in 2008, BioPhorum now comprises over 135 manufacturers and suppliers deploying their top 6,000 leaders and subject matter experts to work in nine focused Phorums, articulating the industry's technology roadmap, defining the supply partner practices of the future, and developing and adopting best practices in drug substance, fill finish, process development and manufacturing IT. In each of these Phorums, BioPhorum facilitators bring leaders together to create future visions, mobilize teams of experts on the opportunities, create partnerships that enable change and provide the quickest route to implementation, so that the industry shares, learns and builds the best solutions together.

1.0

Introduction

Plasmids release specifications are critical to the manufacture of many cell and gene therapy (CGT) products but current guidance defining expectations for release of plasmids as a starting material is limited. With the aim of reaching a consensus on test attributes and release requirements for plasmids and the bacterial master cell banks (MCBs) used to produce them, the BioPhorum CGT Raw Materials Plasmids Release Specifications team collaborated to propose a platform framework to stimulate a broader industry discussion. By focusing specifically on attributes for release testing of plasmid *E. coli* MCBs and plasmid DNA to manufacture viral vectors for delivery of CGTs (e.g. adeno-associated virus or lentivirus), the team's paper published at end of 2020, *Raw Materials: Cell and gene therapy critical starting material: a discussion to help establish release specifications for plasmids and the bacterial master cell banks used to produce them*[2], set out an approach proposing methods and acceptance criteria. The goal of this work was to solicit feedback on the proposed testing practices for plasmid MCBs and plasmid DNA and has since been shared via the BioPhorum website and at conferences. A confidential survey which accompanied the paper invited comment from BioPhorum members and non-members on the specific tests and overall proposed framework. BioPhorum members were also encouraged to provide feedback to the team in group meetings that took place after the initial paper was published.

Alongside this work, in November 2020, members of another CGT Raw Materials team published *Raw Materials: Perspectives on raw and starting materials risk assessment for cell and gene therapy (CGT) processes*[3] discussing a risk-based approach to sourcing and using raw and starting materials for CGT manufacturing processes. It included a case study in the form of a simple risk assessment for plasmid DNA used as part of a CGT process. Since the original paper, the EMA has given specific guidance that plasmids should be made using GMP conditions[4]. This paper supplements the original paper with an example risk assessment to support evaluations for GMP plasmids.

Supplementary to these two previous papers[2, 3] this paper shares knowledge obtained from industry feedback and recent BioPhorum member discussions that may complement ongoing efforts in the wider CGT field to advance release specifications for plasmid MCBs and plasmid DNA and to de-risk the plasmid supply chain.

2.0

Plasmid release specifications – response to CGT industry feedback

The scope of the initial paper[2] was strictly limited to plasmid *E. coli* MCBs and plasmid DNA used as part of CGT manufacturing processes, with multiple steps between the plasmid itself and any material dispensed to the patient. It excludes any plasmid that is directly administered (i.e. injected) into patients or used as a drug substance.

Current mRNA-based vaccines, such as the SARS-CoV-2 vaccines manufactured by Pfizer and Moderna, are produced in a similar manner to mRNA-based gene therapies. That is, a plasmid is linearized, and mRNA is generated as the drug substance. The difference is that mRNA-based vaccines are present in the system for a brief period, are unable to incorporate into human DNA, do not alter DNA, and function as a vaccine against an infectious disease. Whereas mRNA-based gene therapies are intended "to modify the genetic materials of cells" (USP <1047>)[5] and by integration into the genome to insert, delete, or alter the patient's endogenous DNA. It is likely that the concepts described in this paper could also be applied to plasmids used to generate mRNA-based vaccines.

The original paper sets out an approach to platform testing that is presented in a table (Table 1). It summarizes the category of assays (i.e. identity, purity and/or potency), attributes, and suggests methods and acceptance criteria. A copy of the full table is provided in Appendix 1 of this paper. For some attributes, the table presents multiple methods that could be used to assess them. A confidential survey invited BioPhorum members and non-members to give their opinions and thoughts on the suggested approach. In this paper, the team summarizes specific feedback from the CGT community as well as supplementing the original content by discussing some additional points to promote further conversation and industry consensus.

Identity testing and cross contamination (plasmid MCBs and plasmid DNA)

Per Table 1, multiple methods are suggested that could be used to assess identity and cross contamination of plasmid *E. coli* MCBs. Verification of identity is a US Food and Drug Administration (FDA) requirement for any starting material before it is used in a drug manufacturing process[6]. Identity testing methodologies are used to confirm or verify the identity of a material: "Identification procedures should be able to discriminate between materials similar in molecular structure. The lack of specificity of a single technique may be compensated by other supporting analytical procedure(s) or an application of an additional identification technique." (USP <197>)[7]

Plasmid identity testing is performed upon release from the supplier, and by the user prior to use (release panel testing from the supplier/manufacturing department and acceptance testing by the client or verification testing immediately before use in manufacturing). The supplier may use sequencing and/or restriction digest mapping as methods of identity when performing quality control (QC) release testing. In general, the supplier should perform sequencing on the entire plasmid sequence compared to a reference sequence; use of restriction enzyme digestion may be helpful as a qualitative guide.

Table 1 also suggests Sanger sequencing as an alternative method to confirm identity and cross contamination. However, it is recognized that traditional Sanger sequencing may be difficult. Special method adaption is needed due to the complex challenges arising from the repetitive nature of sequence regions present in some plasmid DNA (i.e. repeated long terminal repeat (LTR) and inverted terminal repeat (ITR) sequence regions) and DNA templates (e.g. long stretches of poly(A) sequence regions present in mRNA-based gene therapies). Supplementary to the original paper, the team recommends next-generation sequencing (NGS) techniques if available, which have enhanced capacity to sequence repetitive regions on these sections of plasmid DNA, or to perform a restriction digest that cleaves within the LTR or ITR sequences to demonstrate sequence integrity. The correct process will be plasmid dependent and should be developed in partnership with sequencing data.

The user may also perform either sequencing or restriction digest mapping for release into their process. However, the user may choose to perform sequencing on a smaller, unique section of the plasmid, such as the gene of interest, to confirm identity.

It is technically possible that sequencing techniques can be used to determine whether the intended plasmid has been contaminated by another unwanted plasmid, perhaps through cross contamination during manufacture. If the amount of contamination is sufficiently high, then NGS techniques would likely be able to detect the interfering sequence. Sanger sequencing could technically also be used for this purpose, but due to its lower sensitivity it is less likely that this technique could uncover cross contamination by another unwanted plasmid. Because current sequencing techniques are not capable of identifying very small amounts of plasmid contamination without polymerase chain reaction (PCR) amplification, it is recommended that in-process controls to prevent contamination are in place at the supplier.

For plasmids that are manufactured in compliance with GMP, segregation controls aim to assure low risk of cross contamination during production. For plasmids manufactured in the R&D environment (for early research purposes for example), quality level segregation standards are generally considered less rigorous, and therefore the potential contamination risks greater – robust processes should be in place to mitigate these risks.

As well as contamination by another plasmid, host cell contaminants may be present, such as co-purified host cell RNA, genomic DNA, protein, or endotoxin. Standalone tests for these are therefore required, e.g. qPCR for genomic DNA. Starting material homogeneity is also important.

In-process testing for plasmid isoform purity typically consists of monitoring for denatured plasmid, tracking of percent supercoiling (which may result from the same host plasmid) and identification of target vs non-target plasmid.

Plasmids should be treated as a critical starting material, and the team recommends a risk assessment on the process, to consider at what stage an error would be spotted, and whether sufficient manufacturing and or quality controls are in place.

Identity testing and contamination screening of the MCB itself is also critical. Gram stain analysis, use of selective media, and/or API gallery methods may be performed. If possible, however, 16S rRNA ribosomal sequencing of host cells provides an alternative to all three of these methods.

Appearance testing (plasmid DNA)

Per Table 1, the team proposes visual inspection to test plasmid DNA for appearance upon release and recommends that appearance should be colorless and free of particulate material. For added clarification, since the plasmid in this case is not being injected into a patient directly, consideration of microscopic particulates is not required, and such methods are excluded from the proposed release platform.

Testing for DNA homogeneity (plasmid DNA)

Per Table 1, the team proposes capillary gel electrophoresis (CGE), agarose gel electrophoresis (AGE), densitometry, or HPLC as possible methods to assess DNA homogeneity upon release of plasmid DNA. Feedback received by the team highlights that % supercoiled may impact potency or transfection efficiency. Percent supercoiling and DNA supercoiling refer to over- or under-winding of a DNA strand. Mathematical expressions are used to describe supercoiling by comparing different coiled states to relaxed B-form DNA. The simpler approach for bacterial MCBs and plasmids is to record the percentage of DNA that is in a supercoiled state as determined by CGE, AGE, densitometry, or HPLC. Supercoiled plasmid content has traditionally been evaluated as a measure of quality and stability over time. Having a high supercoiled content for upstream CGT uses may or may not be necessary for plasmids as a starting material. Having a higher supercoiled specification results in additional downstream processing, increased costs, and reduction of overall plasmid yield at suppliers. Users should understand the impact of this specification to optimize the cost and timeline of the overall process. Current thinking is that the need for a certain percentage of supercoiled content should be determined based on the user's needs.

The consensus is that the percentage of supercoiling is related to transfection efficiency and productivity, but the exact relationship is not clear. The team proposes a collaborative approach to collecting sufficient data to understand the correlation between supercoiling and transfection efficiency. BioPhorum processes offer this possibility.

Testing for residual DNA (plasmid DNA)

Table 1 proposes quantitative PCR (qPCR) to assess residual host DNA present in plasmid DNA upon release; however, digital PCR (dPCR) technology has become widely accepted in the pharmaceutical field with many applications. Since the original publication, the team has considered dPCR for the measurement of contamination by other host cells, by targeting the rRNA gene.

Currently, there is no standard tool for performing the assay. Further, there is no agreed methodology to interpret the data that dPCR assays generate. Therefore, use of dPCR to document and record residual DNA contamination has been used cautiously within the CGT field. There is an opportunity to build industry consensus so that the dPCR assay can be used, and the data interpreted in a standard way across industry.

Testing for residual host RNA (plasmid DNA)

In Table 1, use of high performance liquid chromatography (HPLC) or SYBR Gold™, are offered as potential methods to test plasmid DNA for residual host RNA in addition to qPCR. While it is generally accepted that measurement by HPLC may provide superior accuracy, precision, and sensitivity to quantify residual host RNA, alternative methods for quantification of residual host RNA may be advantageous, particularly for plasmid suppliers that may not have HPLC capability. Fluorescent gel staining methods may be suitable as an alternative to HPLC. SYBR Gold for example, developed after SYBR Green I and II, provides sensitive fluorescent gel staining.

In standard AGE applications, SYBR Gold™ is highly selective for RNA, double-stranded DNA and single-stranded DNA. This stain is used for RNA detection in a semi-quantitative manner when compared to a known RNA standard, commonly targeting a final specification of <2% to <5% residual host cell RNA in the final plasmid DNA deliverable. SYBR Gold is one of the more sensitive stains for imaging, which allows lower testing limits down to 20 ng RNA in a 5 µg plasmid DNA sample.

One drawback of SYBR Gold is its high selectivity for DNA as well as RNA. This is only a challenge if there are plasmid DNA low molecular weight species that co-migrate with RNA, or very small non-target DNA species generated during plasmid DNA manufacture and present in the final deliverable product. Protocols may use a plasmid DNA standard gel well which includes RNase and is subtracted from the standard plasmid DNA sample gel well and compared to the RNA standard gel well to address small DNA fragments for an RNA-only comparison[8, 9, 10].

Testing for sterility or bioburden (plasmid DNA)

Table 1 recommends testing by USP<71> direct inoculation or membrane filtration. Feedback received since the original publication highlights that bioburden rather than sterility may be acceptable in some circumstances and the team acknowledges that unique sterility or bioburden requirements for plasmids may be defined through a risk-based approach, based on where and how the plasmids are added to the user's processes.

If plasmids are required to be sterile (e.g. when added to an aseptic manufacturing process without pre-filtration), compendial sterility testing should be performed by the supplier, but reduced volumes may be used per 21 CFR Parts 600, 610, and 680 *Amendments to Sterility Test Requirements for Biological Products*[11] and ICH Q5D *Derivation and characterisation of cell substrates used for the production of biotechnological/biological products*[12]. Relevant plasmid manufacturing unit operations/sterilization controls should be designed to ensure sterility of the final plasmid product, as outlined in Section 9.5.3 of Eudralex Volume 4 *Guidelines on Good Manufacturing Practice specific to Advanced Therapy Medicinal Products*[13] and following the requirements of relevant compendial chapters.

As discussed in the 2021 EMA document *Questions and answers on the principles of GMP for the manufacturing of starting materials of biological origin used to transfer genetic material for the manufacturing of ATMPs*[4], for starting material where a low bioburden specification may be acceptable, the bioburden acceptance criteria need to be defined by the user based on internal procedures, hold times of the low bioburden material before sterile filtration, and possible microbial growth during hold time.

Either criteria (sterility or low bioburden) should be verified by the user as part of plasmid qualification and/or incoming release, based on criticality of microbial control attributes and assessment of existing controls.

Testing for residual kanamycin (plasmid DNA)

Plasmids containing kanamycin resistance gene (e.g. *nptII*) and other antibiotic resistance genes are not uncommon in commercial production and allow for a practical cell selection process of target cells over plasmid-free cells. Kanamycin is an aminoglycoside that binds the 30S ribosomal subunit and causes mistranslation in bacterium. Often used at working concentrations of 50–100 µg/mL in cell selection, toxicity to human health and to non-resistant cell cultures necessitates some level of control and evaluation in raw material plasmid products.

Limits of detection down to 2 ng/mL in enzyme-linked immunosorbent assays may be suitable to demonstrate clearance and elimination of residual kanamycin.

When using other antibiotic or other cell-selective agents, the effects of toxicity or sensitization on the patient should be considered.

3.0

Plasmid release specifications – discussion and future outlook

The following points were not discussed in detail in the original paper but have since been discussed by the team and are included to stimulate further conversation and promote industry consensus.

Elemental, extractable, and leachable impurities

The impact some elemental impurities may have on the integrity of DNA is well documented, as well as the complex interaction between materials and the closures they are stored in. These elements must be taken into consideration in the process development of raw material plasmids in the manufacture and packaging of the source material.

On the user side of transduction, the number and extent of the elemental, extractable, and leachable impurities associated with the raw material plasmid and its closure can make process development challenging. However, it may be prudent to consider the overarching critical quality attributes. In the scope of upstream manufacturing, it may be practical to assess the integrity of the plasmid material (e.g. supercoiling, percentage of nicking, identity, integrity) as an orthogonal measure of the effect of any of these impurities exerted on the material. Further exhaustive effort in screening and characterizing these trace impurities in the plasmid material may not prove fruitful in the overall scope of manufacturing, considering the associated steps and analytical methods utilized downstream[14].

There has been considerable work in BioPhorum on the topic of extractables and leachables for the entire pharmaceutical industry. The material and tools published by this group are freely available on the BioPhorum website[15].

A collaborative approach is recommended to develop a validated methodology and data collection to understand the impurities that may be present and the potential impact. The processes of BioPhorum offer this possibility.

Significant changes

Significant changes for plasmids may be regarded as those that impact the quality, safety, and efficacy of the plasmids. Considerations from the International Council for Harmonization (ICH) Q5E *Comparability of biotechnological/biological products* may be applied to demonstrate that changes to the plasmid manufacturing process do not adversely impact the quality, safety, and efficacy of the plasmids. The rigor around evaluating comparability should be on par with the treatment modality the plasmids are used in and in proximity to the final drug product/patient, phase of development and associated manufacturing/clinical experience and history.

The following criteria may be considered when evaluating the potential impact of a change:

- The manufacturing step where the change is introduced and the impact of the change to the validated state (if applicable) of a plasmid manufacturing process
- The potential impact of the change on the purity as well as on the physicochemical and biological properties of the plasmid
- The availability of suitable analytical techniques to detect/measure quality attributes that may be impacted by the change
- The relationship between impacted quality attributes and safety and efficacy, based on overall nonclinical and clinical experience.

The outcome of an evaluation may help the end-user determine the potential impact/severity of the change and define their change management strategy accordingly.

Stability studies

Stability study design, requirements, and interpretation of results for plasmids can be acquired directly from ICH quality guidelines including, *Q1A (R2) Stability testing of new drug substances and products*, *Q1D Bracketing and matrixing designs for stability testing of new drug substances and products*, *and Q1E Evaluation of stability data*. While these ICH guidelines are recommendations intended for drug substances and drug products, the concepts and suggestions can be applied to plasmids used as starting materials in CGT.

Stability testing should be done at the desired condition; -80°C or liquid nitrogen (-196°C). Testing is typically done at T0, every three to six months for the first two years and annually thereafter. Accelerated stability testing is possible. The following tests are typically stability indicating; UV spectrophotometry A260/A280, homogeneity (CGE, AGE, densitometry or HPLC), microbial testing (e.g. container closure or sterility or bioburden, as appropriate) and pH testing. The set of tests used may be more comprehensive or abbreviated at different time points and will be documented in the stability plan.

4.0

Re-evaluating plasmid risks

Alongside work to define a release platform for plasmid MCBs and plasmid DNA, members of the CGT Raw Materials team published *Raw Materials: Perspectives on raw and starting materials risk assessment for cell and gene therapy (CGT) processes*[3] in November 2020 discussing a risk-based approach to sourcing and using raw and starting materials for CGT manufacturing processes. In this original publication, a risk assessment for a plasmid that is used to transfect cells and generate viral vectors for AAV processes is shown as a case study example in Table 2 in the original publication[3]. This table is reproduced in Appendix 2. Since the original paper was published, the EMA has given specific guidance that plasmids should be made using GMP conditions[4]; plasmid user needs are changing as technology continues to evolve. This supplementary paper therefore aims to update the example case study risk assessment presented in the original publication by evaluating two potential suppliers of GMP plasmid (see Table A). For a risk assessment which can be applied broadly across the biopharmaceutical industry reference to *Raw material risk assessments: A holistic approach to raw material risk assessments through industry collaboration*[16] is recommended.

Table A considers an up-to-date comparison between two suppliers (Source manufacturer A and Source manufacturer B). In this scenario, the plasmid is used to transfect cells and generate viral vectors for AAV processes, a common CGT process[17, 18]. These two items can be sourced from a multitude of suppliers, so a risk-based comparison between suppliers is recommended. In the example in Table A, three key criteria were used to differentiate between the suppliers:

- The availability of pharmaceutical grade GMP plasmid
- Sterility vs bioburden control and the use of Sanger sequencing
- User needs.

The degree to which a supplier can meet these requirements and provide documentary evidence to support this is central to the use of plasmids in the CGT industry.

It is expected that the content of this table will change and adapt as further data is released, for example on use of NGS, new processing developments or expansion of knowledge of extractables and leachables. The table is a guide to aid discussion and further conversation across the industry, and this topic and ongoing work will be revisited and updated as appropriate.

Table A: Example Case study 3 Plasmid

Material name: Plasmid xxxx		SAP #				
CAS # N/A		Legacy #				
Ancillary		Item name	Plasmid		Plasmid	
		Catalog #				
		Supplier	Typically re-packager		Typically re-packager	
		Manufacturer	Source manufacturer A		Source manufacturer B	
		Location				
		Disposition	Selected Source		Potential Source	
Criteria	**Source for assessment**	**Weight**	**Scoring (1,3,9)**	**Sources for score**	**Scoring (1,3,9)**	**Sources for score**
Patient exposure	UR	9	1	Ancillary raw material	1	Ancillary raw material
Process robustness	UR – Impact to product quality	7	9	Key role in transfection, viral production and DNA transcription	9	Key role in transfection, viral production and DNA transcription
	UR – Impact to process					
	MA – Manufacturing complexity and impurities					
RM variability/ complexity	MA – Origin, composition, structural complexity	7	1	Full Sanger/NGS sequencing	3	Restriction digest
	MA – Manufacturing complexity and impurities					
Origin and impurities	MA – Origin, composition, structural complexity	3	3	Fermented	3	Fermented
	MA – Manufacturing complexity and impurities					
Regulatory impact/compendia compliance	UR – Regulatory/compendia requirements	5	1	Pharmaceutical grade available	3	No pharmaceutical grade available
	MA – Origin, composition, structural complexity					
	MA – Analytical complexity/ compendia status					
	SC – Supplier material grade					
Microbial restrictions/ characteristics	UR – Microbial restrictions	3	1	Sterile	3	Non-sterile, bioburden control
	MA – Microbial characteristics					
Material shelf life and stability	MA – Material shelf life and stability	1	1	Stable, data on file	3	Stable, supplier does not have data
	SC – Supplier technical capability					
Material acceptance	UR – Material acceptance requirements	3	1	Fit-for-use testing on CoA	3	Sanger sequencing not performed, may require additional confirmatory testing
	MA – Analytical complexity/ compendia status					
	SC – Supplier technical capability					

Table A: Example Case study 3 Plasmid (continued)

Material name: Plasmid xxxx		**SAP #**				
CAS # N/A		**Legacy #**				
Ancillary		**Item name**	**Plasmid**		**Plasmid**	
		Catalog #				
		Supplier	Typically re-packager		Typically re-packager	
		Manufacturer	Source manufacturer A		Source manufacturer B	
		Location				
		Disposition	**Selected Source**		**Potential Source**	
Criteria	**Source for assessment**	**Weight**	**Scoring (1,3,9)**	**Sources for score**	**Scoring (1,3,9)**	**Sources for score**
Supply chain	SC – Supplier quality system performance	3	3	Established supply chain, no secondary site	3	Established supply chain, no secondary site
	SC – Continuity of supply					
	SC – Supplier relationship					
	SC – Supplier technical capability					
Inventory management	MA – Material handling requirements	1	1	Off-the-shelf, no long lead time, no safety stock, refrigerated storage	1	Off-the-shelf, no long lead time, no safety stock, refrigerated storage
	SC – Continuity of supply					
Total risk score			**110**		**148**	

5.0

Summary

In this paper, the team build on the previous BioPhorum publication *Cell and Gene Therapy Critical Starting Material: A Discussion to Help Establish Release Specifications for Plasmids and the Bacterial Master Cell Banks used to produce them* to expand CGT industry knowledge in the field of plasmid release specifications and to contribute to parallel efforts within the sector to standardize plasmid release specifications. This paper aims to highlight mitigation of plasmid supply risks through risk assessments.

In the fast-moving field of CGT manufacture, there are likely to be advances in the next few years in the application of science to plasmid release specification. One key area will be the use of NGS for determining identity and contamination control. The team will continue to monitor work in this arena and apply it wherever possible to control of plasmid release specifications.

The relationship between varying degrees of supercoiling, differing plasmid sequences and differing transfection protocols is uncertain but clarification is likely to come soon as data is collected by manufacturing companies.

Since the publication of the initial paper[2], the US Pharmacopoeia has formed an expert panel to draft a new chapter to document and standardize plasmid release specifications where plasmid DNA will be used as a starting material for manufacture of CGTs. Applying a common standard will enable communication and co-operation across the pharmaceutical industry and will simplify communication with regulators. Publication of this common standard is welcomed. In the interim, it is hoped that this paper will assist those using plasmids in CGT manufacturing processes.

Appendix

Appendix 1

Table 1: Proposed plasmid MCB and plasmid DNA testing and release platform[1]

Assay type	Stage	Attribute	Method	Acceptance criteria	BioPhorum industry survey comments	Assay-specific questions
Identity	MCB	Cross contamination	Bacterial colony morphology	Uniform colonies, no visible contaminants		
Purity	MCB	Lytic phage contamination	Plaque formation on lawn	Absence of phage		
Identity	MCB	Cross contamination	Gram stain analysis	Identity confirmed	All might not be required; 16S rRNA sequencing may be used in addition or to replace these three methods	
Identity	MCB	Cross contamination	Analytical profile index (API)			
Identity	MCB	Cross contamination	Selective media			
Identity	MCB	Cross contamination	EtBr stained AGE	Co-migrates with reference DNA or size versus a supercoiled marker	Not always required; determines size only Purified material is needed for electrophoresis	
Identity	MCB	Identity and cross contamination	Restriction digest plus EtBr stained AGE	Matches client-supplied materials/ reference pattern	Purified material is needed for electrophoresis	What is the ideal number of bands to have for an identity method? What is the ideal number of restriction enzymes to use for an identity method? Do you have regions of the plasmid whose presence has to be determined by restriction enzyme digestion (critical areas)?
Identity	MCB	Identity and cross contamination	Double stranded primer walking (Sanger sequencing)	Identical to client supplied sequence or reference material	Purified material is needed for sequencing Key to identification Methodology changes as move through the different stages Exact practice may vary in terms of coverage, base call quality, etc.	What sequencing coverage (1X, 2X, bidirectional, etc.) do you require for inverted terminal repeats ITRs? What sequencing coverage (1X, 2X, bidirectional, etc.) do you require for the gene of interest (GOI)? What sequencing coverage (1X, 2X, bidirectional, etc.) do you require for, other plasmid sequences, e.g. backbone? Do you routinely streak to a single colony to ensure monoclonality prior to sequencing? This is usually performed twice (2 rounds).
Purity	DNA	Purity	UV spec A260/A280	1.8–2.0		
Purity	DNA	Appearance	Visual inspection	Free of particulate material and colorless	Development of a turbidity and colorimetry specification (i.e. a method with a quantifiable output) is desirable Free of particulate; may vary by process	Are there any instances where specification of 'free of particulates' is not needed?

Table 1: Proposed plasmid MCB and plasmid DNA testing and release platform[1] (continued)

Assay type	Stage	Attribute	Method	Acceptance criteria	BioPhorum industry survey comments	Assay-specific questions
Potency	DNA	Concentration	UV Spec A260	Concentration will be determined by program +/- 10%	Some methods require +/-1% Actual concentration cannot be specified, e.g. larger plasmids may require a higher concentration	Do you require a minimum concentration? What is the desired accuracy of your concentration method?
Purity/ Potency	DNA	Concentration DNA homogeneity	Capillary gel electrophoresis (CGE) or Agarose gel (AGE) Densitometry or HPLC	> 80–85% supercoiled	The % supercoiled required may change by process; the sum of monomer and dimer is required in some circumstances	What is the preferred standard method to determine DNA homogeneity? How does % supercoiled relate to potency or transfection efficiency or productivity? (Discuss)
Purity	DNA	Endotoxin	Various	<20–<100 EU/mg	Recommendation to lower the level of endotoxin present. The kinetic chromogenic assay, EndoSafe portable testing (PTS) was considered; not all members had experience to recommend as a standard Recommendation to harmonize units to EU/mg	
Identity	DNA	Identity	EtBr stained AGE	Co-migrates with reference DNA or size versus a supercoiled marker	Not a universal requirement	
Identity	DNA	Identity	Double-stranded primer walking Sanger sequencing	Identical to client supplied sequence or reference material	Exact practice may vary in terms of coverage, base call quality, etc.	Are you using next generation sequencing (NGS) for characterization (additional confirmation)?
Identity	DNA	Identity	Restriction digest plus EtBr stained AGE	Matches client-supplied materials/ reference pattern		
Purity	DNA	Residual host DNA	Quantitative PCR	<1–5%	<5% may be acceptable in certain circumstances Suggest standardization of units to fg/uL	Are we ready to transition to digital PCR as an industry?
Purity	DNA	Residual host protein	MicroBCA (bicinchoninic acid assay)	<1–2%	<2% may be acceptable in certain circumstances	
Purity	DNA	Residual host RNA	HPLC or SYBR Gold	<1–5%	In some cases, SYBR Gold was not the preferred method (semi-quantitative)	Should we standardize on HPLC?

Table 1: Proposed plasmid MCB and plasmid DNA testing and release platform[1] (continued)

Assay type	Stage	Attribute	Method	Acceptance criteria	BioPhorum industry survey comments	Assay-specific questions
Purity	DNA	Sterility or Bioburden	USP <71> (8) Direct inoculation or membrane filtration	No growth	Bioburden rather than sterility may be acceptable in some circumstances One scenario may be the use of a test like BacT/ALERT; comparability study data and acceptance from regulators will be required	ICH Q15D allows reduced sampling for starting materials. How do you currently reduce sample load (impact on the product batch volume)? What are your views on rapid sterility testing in lieu of USP<71>?
				Direct inoculation no bacteriostasis/ fungistasis	Not required for bioburden Membrane filtration is also acceptable	
Purity	DNA	Residual Kanamycin	ELISA or HPLC?		Kanamycin is the most used Recommend not to use ampicillin	What is your preferred method for quantifying residual kanamycin; ELISA or HPLC? What level of residual kanamycin is acceptable? (ng/mL)
Purity	DNA	Mycoplasma	USP <63> (9)	None detected	Process dependent	Would you be supportive of a move to mycoplasma rapid-release testing using PCR, if a validated method was available?
Identity	DNA	pH	USP <791> (10) (potentiometric)	Formulated properly, no gross formulation errors in stability	Process dependent	
Identity	DNA	Osmolality	USP <785> (11), Ph.Eur. 2.2.35 (12) (vapour pressure/ dew point and freezing point depression)	Formulated properly, no gross formulation errors in stability	Process dependent	

Appendix 2

Table 2: Case study 2 Plasmid[2]

Material name: Plasmid	SAP #					*Considerations and actions: elemental impurities, filing strategies, quality agreement and change control agreement, siting decisions (CMO versus internal)*
CAS # 9002-98-6	Legacy #					
Ancillary	Item name	Plasmid		Plasmid		
	Catalog #					
	Supplier	Typically re-packager		Typically re-packager		
	Manufacturer	Source manufacturer 1		Source manufacturer 2		
	Location					
	Disposition	Selected Source		Potential Source		
Criteria	**Source for assessment**	**Weight**	**Scoring (1,3,9)**	**Sources for score**	**Scoring (1,3,9)**	**Sources for score**	**Mitigation plans**
Patient exposure	UR	9	1	Ancillary raw material	1	Ancillary raw material	
Process robustness	UR – Impact to product quality UR – Impact to process MA – Manufacturing complexity and impurities	7	9	Key role in transfection, viral production and DNA transcription	9	Key role in transfection, viral production and DNA transcription	
RM variability/ complexity	MA – Origin, composition, structural complexity MA – Manufacturing complexity and impurities	7	1	Full NGS sequencing	3	Restriction digest	
Origin and impurities	MA – Origin, composition, structural complexity MA – Manufacturing complexity and impurities	3	3	Fermented	3	Fermented	
Regulatory impact/ compendia compliance	UR – Regulatory/compendia requirements MA – Origin, composition, structural complexity MA – Analytical complexity/ compendia status SC – Supplier material grade	5	1	Pharmaceutical grade available	1	No pharmaceutical grade available	
Microbial restrictions/ characteristics	UR – Microbial restrictions MA – Microbial characteristics	3	1	Sterile	1	Sterile	
Material shelf life and stability	MA – Material shelf life and stability SC – Supplier technical capability	1	1	Stable, data on file	3	Stable, supplier does not have data	
Material acceptance	UR – Material acceptance requirements MA – Analytical complexity/ compendia status SC – Supplier technical capability	3	1		1		

Table 2: Case study 2 Plasmid[2] (continued)

Material name: Plasmid			SAP #				*Considerations and actions: elemental impurities, filing strategies, quality agreement and change control agreement, siting decisions (CMO versus internal)*
CAS # 9002-98-6			Legacy #				
Ancillary			Item name	Plasmid		Plasmid	
			Catalog #				
			Supplier	Typically re-packager		Typically re-packager	
			Manufacturer	Source manufacturer 1		Source manufacturer 2	
			Location				
			Disposition	Selected Source		Potential Source	
Criteria	**Source for assessment**	**Weight**	**Scoring (1,3,9)**	**Sources for score**	**Scoring (1,3,9)**	**Sources for score**	**Mitigation plans**
Supply chain	SC – Supplier quality system performance SC – Continuity of supply SC – Supplier relationship SC – Supplier technical capability	3	3	Established supply chain, no secondary site	3	Established supply chain, no secondary site	
Inventory management	MA – Material handling requirements SC – Continuity of supply	1	1	Off-the-shelf, no long lead time, no safety stock, refrigerated storage	1	Off-the-shelf, no long lead time, no safety stock, refrigerated storage	
Total risk score			**110**		**126**		

Definitions

Term	Definition
Supplier	For the purposes of this document, and in alignment with the ISO ancillary materials standard[1], 'supplier' can mean plasmid manufacturer, plasmid supplier, etc.
User	For the purposes of this document, and in alignment with the ISO ancillary materials standard[1], 'user' can mean drug developer, sponsor, end-user, DP manufacturer, etc.

References

1 **ISO/DIS 20399 Biotechnology – Ancillary materials present during the production of cellular therapeutic products and gene therapy products**

2 BioPhorum *Raw Materials: Cell and gene therapy critical starting material: a discussion to help establish release specifications for plasmids and the bacterial master cell banks used to produce them* (2020)

3 BioPhorum ***Raw Materials: Perspectives on raw and starting materials risk assessment for cell and gene therapy (CGT) processes* (2020)**

4 European Medicines Agency EMA/246400/2021 *Questions and answers on the principles of GMP for the manufacturing of starting materials of biological origin used to transfer genetic material for the manufacturing of ATMPs* (2021) **https://www.ema.europa.eu/en/documents/other/questions-answers-principles-gmp-manufacturing-starting-materials-biological-origin-used-transfer_en.pdf**

5 US Pharmacopeia Standards for Cell and Gene Therapy <1047> Gene Therapy Products

6 US FDA 21 Code of Federal Regulations Part 610: *FDA General Biological Products Standards*

7 USP <197> Spectrophotometric Identification Tests

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11 21 CFR Parts 600, 610, and 680 *Amendments to Sterility Test Requirements for Biological Products*

12 European Medicines Agency ICH Q5D *Derivation and characterisation of cell substrates used for the production of biotechnological/biological products*

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