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CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON
Gene therapy analytics and CMC

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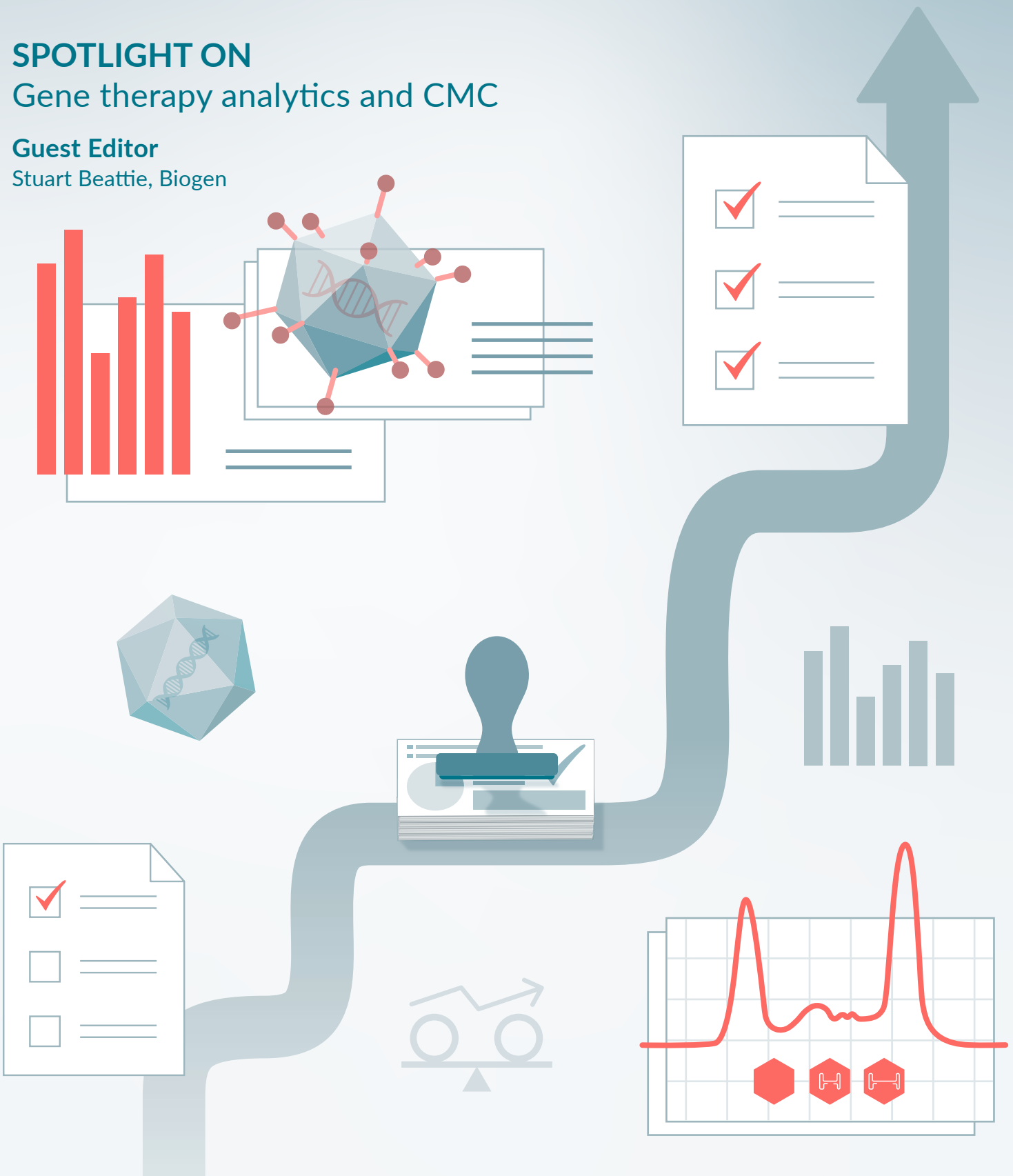

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REGULATORY PERSPECTIVE

Clinical trial applications for investigational medicinal products that contain or consist of genetically modified organisms: industry experiences under the European Union Clinical Trial Regulation (536/2014)

Stuart G Beattie, Nathalie Lambot, Jacquelyn Awigena-Cook, Martin O'Kane, Caroline Correas, Ine de Goeij, Julien Romanetto, Annelie Persson, and Pär Tellner

The survey reported upon here provides an up to date understanding of industry experiences submitting national GMO applications since the CTR has been in application (January 31, 2022). The survey shows how time- and resource-intensive applications seeking authorizations for use of investigational medicinal products that contain or consist of genetically modified organisms (GMO-IMPs) to EU Member States continue to represent a significant challenge for developers. EU Member State GMO competent authorities presently apply differing interpretations of the European Commission Directives for Deliberate Release of GMOs and/or the Contained Use of GMOs. Survey feedback highlights how varied the different EU Member State GMO competent authority procedures and assessment timeframes are, with differences in adaptation to the timelines dictated by the Clinical Trial

Regulation (CTR). Lengthy and uncertain timelines associated with EU Member State GMO competent authority procedures were indicated to have led sponsors to have looked to other regions (USA, Canada, and Australia) to host clinical trials with GMO-IMPs. The benefits of a single clinical trial application submission under the CTR are considerably diminished due to different national GMO procedural and documentation requirements and a lack of formal alignment of timelines between CTA and GMO procedures. EFPIA welcome the proposed improvements for regulation of GMO medicines through revision of the EU General Pharmaceutical Legislation.

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Regulation (EU) No 536/2014 of the European Parliament and of the Council of April 16, 2014 on Clinical Trials on Medicinal Products for Human Use, referred to as the Clinical Trial Regulation (CTR) has been in application since January 31, 2022 [1,2]. The CTR replaced the Clinical Trials Directive (CTD, 2001/20/EC) [3,4]. Under the CTR, a single electronic clinical trial application (CTA) dossier is submitted to all the Member States involved in the trial, via the European Clinical Trials Information System (CTIS) portal [5]. Since January 31, 2023, all initial applications of interventional clinical trials (CT) for investigational medicinal products (IMPs) are required to be submitted under the CTR. Ongoing trials approved under CTD will have to transition to the CTR before January 30, 2025 [6].

Gene therapies are treated as genetically modified organisms (GMOs [7]) and national GMO competent authorities require that Sponsors provide an environmental risk assessment (ERA) as part of a GMO application. Authorization for use of a GMO Investigational Medicinal Product (GMO-IMP) is required before a clinical trial can commence. As detailed in the findings of this survey, for a couple of member states, approval of the GMO-IMP is required before the CTA can even be submitted. GMO applications are under the purview of each EU Member State's GMO competent authority and national GMO competent authorities nearly always differ from the national health authorities that are involved with assessment of the CTA (under the CTR [8]). GMO requirements are currently applied at the national level and are interpreted differently, with more often than not, GMO assessment timeframes that are not aligned to CTAs, as defined under the CTR.

The European Union (EU) legislation prescribes that clinical trials with investigational products containing or consisting of GMOs comply with either Directive 2001/18/EC on the Deliberate Release (DR) into the environment of GMOs [7], or with Directive 2009/41/EC on the Contained Use (CU) of GMOs [9]. Whilst a 2008 EMA guideline [10] describes the scientific principles and methodology for an ERA of a gene therapy, documentation and submission requirements differ significantly depending on whether the Directive for DR or CU is applied for the specific GMO in the specific EU Member State. Both GMO Directives are transposed into national law with variations and are applied in different manners by national competent authorities in each Member State. Annex II of the Directive for DR lays out the principles for the ERA, whereas, if a Member State considers the clinical testing of a gene therapy to be CU, then only a somewhat limited

risk assessment of containment measures is required (although there are clinical site-specific requirements under CU, which can be burdensome). Interpretations of the two Directives and GMO framework are applied inconsistently, leading to different assessment processes, timelines, and outcomes across the EU.

An industry survey was previously conducted in 2020 and identified then how GMO approval timelines were highly variable, often within the same Member State [11]. Some countries granted the majority of approvals within 60 days, as reported for France and Belgium; whilst other countries took longer to approve, as reported for Spain, Italy, Germany, and the Netherlands. In 2020, 32% of GMO approvals were granted after approval of the CTA, indicating that the GMO approval process has caused significant delays to the initiation of clinical trials. Also reported were how, despite the adoption of Common Application Forms (CAFs [12–14]) and Good Practice Documents [15,16] across EU Member States, additional data and forms were requested by competent authorities for nearly all GMO submissions, often duplicating information already provided in CAFs, with additional local language requirements.

The survey reported upon here provides an up to date understanding of industry experiences submitting national GMO applications, since the CTR has been in application (January 31, 2022). Survey feedback highlights how varied the different EU Member State GMO competent authority procedures and assessment timeframes continue to be. Due to differing application and interpretation of DR and CU Directives, together with differing procedures, data requirements, and assessment timeframes across EU Member States, the time- and resource-intensive GMO applications continue to represent a significant hurdle for Sponsors. Despite the adoption of harmonized (common application) forms for GMO applications, the benefits of a single CTA submission under the CTR are diminished since CTA coordination under the CTR does not facilitate multi-Member State GMO applications. There is currently no mechanism for a single submission of GMO applications for multinational trials. Lengthy and uncertain timelines associated with some EU Member State GMO competent authority procedures were indicated to have led sponsors to have looked to other regions (USA, Canada, and Australia) to host clinical trials with GMO-IMPs.

SURVEY

A survey, comprising 11 questions, was conducted between July 3 and September 29, 2023.

The objective of the survey is to understand industry experiences when submitting a CTA for a GMO-IMP, since the CTR (536/2014) has been in application, that is, since January 31, 2022.

The survey was distributed amongst members of The European Federation of Pharmaceutical Industries and Associations (EFPIA), The European Confederation of Pharmaceutical Entrepreneurs (EUCOPE) and The European Association for Bioindustries (EuropaBio). Returned survey responses were aggregated and processed anonymously.

As below, the survey is divided into two sections worth of questions:

- Section 1: submitting CTAs under CTR (for GMO-IMPs);
- Section 2: GMO procedures at the national level.

Survey responses from 18 members of EFPIA were received, of which 12 completed the survey with direct and relevant experiences of submitting CTAs for GMO-IMPs under the CTR, as outlined below.

The results are shown per survey question, along with further explained context and comment, where relevant.

RESULTS

Section 1: submitting CTAs under CTR (for GMO-IMPs)

Question 1: have you submitted a clinical trial application (CTA) or multiple CTAs with a GMO-IMP (or GMO-IMPs) within the EU since the application of the CTR, January 31, 2022 (regardless of whether submitted through the CTR or through the Clinical Trials Directive)?

There were 18 responses: 7 answered 'no'. 11 answered 'yes'.

It was noted that one respondent had, at that point, not yet submitted the CTA for the GMO-IMP in the EU, despite having submitted a GMO application. This was because one of the EU Member States' GMO competent authority had requested that the GMO application be submitted first, with approval of the GMO application expected prior to submission of the CTA. The respondent's experience regarding the submission of the GMO package is captured in the second part of the survey (question 7 onwards).

Question 2: If not, have you submitted a CTA with a GMO-IMP in another region of the world (outside the EU) since January 31, 2022?

There were 18 responses: 15 answered 'no'. 3 answered 'yes'.

Sub-question: Please list countries submitted to outside of the EU (and number of times a CTA was submitted to each country):

Answer(s): USA (twice); Canada; Australia.

Question 3: If you answered 'yes' (to question 2), did any of the following factors influence your decision not to submit a CTA within the EU?

Please select all that apply and please elaborate where possible.

From the three respondents who answered 'yes' to question 2, the following factors had influenced their decisions:

- Complexity/lack of clarity of CTR procedures: yes (1 response);
- Complexity of national/local application procedures (under the CTR): yes (1 response);
- Complexity of local GMO application procedures: yes (1 response);
- Complexity of GMO interplay/harmonization with CTR: yes (1 response);
- Other (please specify): long and uncertain approval timelines: yes (2 responses).

An Alliance for Regenerative Medicine Sector Report for H1 2022 illustrated how compared to North America and Asia Pacific, 'Europe' (also presumably including UK) accounted for the smallest share of new clinical trials (11%) and was the only region with a bottom-heavy pipeline comprised of later phase trials [17]. In 2022, this was considered to be partly attributable to complex and time-consuming GMO environmental requirements for GMO-ATMPs within the EU.

It is noted how lengthy and uncertain timelines associated with national EU Member State GMO competent authority procedures were indicated to have led sponsors to have selected other regions to include within a clinical trial for the GMO-IMP in development (USA, Canada, and Australia).

- In the USA, for gene therapies, vectored vaccines, and related recombinant viral or microbial products, sponsors benefit from a claim of 'categorical exclusion' that ordinarily applies to clinical studies, allowing an exemption from

the requirements for an environmental assessment under 21 CFR 25.31(e) for Investigational New Drugs (FDA 2015 [18]; FDA CFR Title 21 [19]);

- Canada and Australia also benefit from well-defined procedures and data requirements for application of use for GMO medicinal products, which may also allow for improved forecasting of review and approval timings (compared to that across EU Member States, when undertaking a multiregional clinical trial);
  - Despite well-defined GMO review timelines, Canada and Australia have in recent years undertaken public consultations, with a view to future improvements to the regulation of GMOs;
    - The Canadian Government have recently published what they heard through the stakeholder pre-consultation on the review of Part 6 of the Canadian Environmental Protection Act, 1999 (CEPA) and the New Substances Notification Regulations (Organisms) [20];
    - Similarly, the Australian Government Department of Health and Aged Care National Gene Technology Scheme, Consultation Regulation Impact Statement (CRIS) have published responses (where permission was given [21]).

Question 4: If you submitted an initial CTA for a GMO-IMP within the EU, did you submit according to the Clinical Trial Regulation (CTR, 536/2014) via CTIS, or under the Clinical Trials Directive, via EudraCT? Select which applies and how many CTAs were submitted for each.

There were 11 responses:

- Clinical Trial Regulation (CTR): 5 responses;
- Clinical Trials Directive (CTD): 3 responses;
- Both CTR and CTD (for separate CTAs): 3 responses.

From January 31, 2022 through to January 31, 2023, some sponsors had continued to submit CTAs under the CTD. This highlighted a perceived lack of predictability in CTAs via CTIS (under the CTR) and perceived potential for delays to authorization of the CTA, especially when CTIS was first 'live' and especially for trials with GMO-IMPs which may be perceived as being more complex.

Question 5: If you have submitted a CTA with a GMO-IMP under the Clinical Trials Directive (rather than the CTR) was this due to any of the below reasons? Please select all that apply and elaborate where possible.

- Lack of harmonization of EU Member States in the field of GMO CTAs: no responses;
- Lack of alignment between the local application process and/or timelines for the GMO and the procedure for the CTR: 1 response;
- Other reason not related to GMO requirements: 5 responses;
- Other (please specify):
  - Lack of clarity about operational challenges associated with a submission under the CTR;
  - CTA preparation started prior to the implementation of the CTR and choice of the sponsor to continue with the submission plans under the CTD.

Question 6: If you submitted an initial CTA for a GMO-IMP under the CTR, which countries did you select for your study, and why?

There were 8 responses, where the following EU Member States were selected as countries to undertake a clinical trial:

Austria, Belgium, Czechia, Denmark, Estonia, France, Germany, Greece, Hungary, Italy, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Spain, and Sweden.

The primary reasons that survey respondents provided for selection of the EU listed Member States to host an initial CTA for a GMO-IMP under the CTR were not based on GMO issues.

Instead, access to patient populations and other clinical trial considerations dictated such country selections. Clinical considerations included the following:

- Availability and suitability of a clinical site and access to patients;
- Availability of a Principal Investigator (PI) or investigators and possibility of interaction with such PIs;
- The possibility to apply decentralized point of care manufacturing at the clinical site.

Other survey respondents did provide comment on how the current country-specific GMO requirements adjacent to a CTA have influenced country selection:

- One survey respondent noted that their experience of the GMO requirements within Czechia (formerly known as the Czech Republic) could be a factor for future selection. This is because Czechia requires approval of the GMO application prior to filing of a CTA;
- Another respondent indicated that countries, where prior approval of the GMO application is required to the CTA were then deselected as potential regions to undertake a clinical trial: Belgium, Czechia, and Poland;
- Another survey respondent indicated how prior positive experiences applying for clinical trials of GMO-IMPs, where there were clearly defined GMO requirements have led to reselection of those particular EU Member States as hosts: Germany and Spain.

Sequencing of applications for authorization for use of GMO-IMPs and CTAs

Regarding specific EU Member States requirement that an application for GMO use be approved by the competent authority before submission of a CTA, it is worth noting how this issue (and potential delay to the start to a clinical trial) had been previously identified by the European Commission.

Although not adopted by the European Commission, nor an official position, version 3 of the Q&A document "Medical Products for Human Use Containing or Consisting of GMOs: Interplay between the EU Legislation on Medical Products and GMOs" (October, 2019 [22]) specifically addresses the interplay and sequencing of national GMO applications and a CTA.

Question 1 of the Q&A document asks, *"Is the authorisation under the GMO framework a pre-requisite to the submission of a clinical trial authorisation application?"*

In the answer, it is stated that, *"When the Regulation (EU) No 536/2014 on clinical trials becomes applicable, the prior authorisation under the GMO framework can no longer be a pre-requisite for a valid clinical trial authorisation application."*

The answer to the question further elaborates on how, *"under Article 5(3)(b) of the Regulation, the assessment whether an application dossier is complete is to be done in accordance with the detailed provisions of Annex I.*

In turn, Annex I does not list the prior authorisation under the GMO framework as an element to be considered for the completeness check. Moreover, the assessment of the Member States concerned is limited to the aspects enumerated in Articles 6 and 7 of the Regulation and the lack of GMO authorisation cannot justify a negative decision by a concerned Member State."

And, how that, *"It follows that, under the CTR, an application for clinical trials authorisation cannot be turned down on grounds that the authorisation under GMO framework has not been obtained at the time when the application for clinical trial authorisation is submitted".*

Section 2: GMO procedures at the National Level

Question 8: If you submitted a GMO package of documents, was the review timeline for the GMO package aligned with the CTR timelines?

There were 12 responses:

- 3 answered 'yes'
- 6 answered 'no'
- 3 answered 'not submitted under (the) CTR'

The review and approval timelines, with further detail for each of the following countries, are shown in order of the time taken to review and approve the GMO application:

- France: 15–20 day. All submitted via the 2009/41/EC Directive on the Contained Use of GMOs (hereafter abbreviated to CU) [9];
- Belgium: 20 days via CU;
    - For both the CU procedure and the procedure for the 2001/18/EC Directive on the Deliberate Release of GMOs (hereafter abbreviated to DR [1]) approval of the GMO applications were available prior to approval of the CTA.
- Portugal: 30 day (via CU);
- Greece: 49 day GMO approval;
- Norway: 54 day GMO approval.
- Spain: Via DR, 70, 75, 90, and 120 day GMO approval timeframes were experienced, where approval for use of the GMO-IMPs were available prior to CTA approval;
- Romania: 78 day;
- Germany: 90 day GMO approval (via DR). 120 day CTA approval;
- Belgium: 90 day via application of the DR Directive;
- Italy: 90 day (via CU) earliest approval timeline available;
- Netherlands: 90 day approval, available prior CTA approval;
    - The possibility of an umbrella (GMO) licence application was also noted. Once granted, there is the possibility to apply for a 'copy license', following a theoretical 28 day timeframe [23];
- Czechia: as noted earlier, Czechia requests the GMO submission prior to the CTA submission. This delayed commencement to the clinical trial. 90 day (via CU) and 106-day review timeframes were provided;
- Hungary: 120 day (via DR);

- Sweden: 150 day (via DR) with CTA approved after 99 days. Both the GMO and CTA approval letters were dated identically.
- Poland: as noted earlier, Poland requests the GMO submission prior to the CTA submission. This delayed commencement to the clinical trial. A 165-day timeframe via DR was reported;

It was also reported that for the following countries, a CTA with a GMO-IMP that already has marketing authorization (for a different indication) allowed for national GMO review that was either faster than or aligned to the CTA timeframe: Austria, France, Germany, Italy, The Netherlands, Portugal, Spain, Sweden.

Question 9: If you answered 'no' (to question 8) did the GMO application delay initiation to the clinical trial?

There were 6 responses:

- 3 answered 'yes';
- 2 answered 'no';
- Within the survey window of time, one company could not yet confirm if the GMO application had led to a delay to the start of the clinical trial.

Survey respondents reported that the following EU Member States GMO procedures had led to delayed starts to a clinical trial:

- Austria (1 month delay);
- Czechia (between 6 and 12 months);
- Italy (between 1 and 3 months delay);
- Poland.

No delays were reported for the following countries: Belgium, Denmark, France, Germany, Greece, Norway, Romania, and Spain.

Question 10: Please indicate the number of GMO submissions per modality that your organization has submitted since January, 2022.

The respondents entered once per investigational product:

- IMPs containing genetically modified human cells—modified *ex vivo* using a viral vector (e.g., CAR-T cells modified using a lentiviral, or retroviral vector): 28;
- Gene therapy IMPs consisting of a viral vector intended for direct administration (e.g., adeno-associated viral vector, AAV): 10;
- GMO virus-based vaccine: none;
- IMPs containing other types of genetically modified cells (e.g., genetically modified bacteria): none;
- GMO submissions for other IMPs other than listed above, e.g., human cells genetically modified with non-viral vectors: none.

In line with the latest Gene, Cell and RNA Therapy Landscape Reports, CAR-T therapies dominate the pipeline, with viral vector-delivered therapies in second place [24].

Question 11: Please provide other comments to your experiences submitting a CTA and GMO package for an GMO-IMP since January 31, 2022?

The following answers were provided, with requested elaboration for each national GMO competent authority/liaison office). Provided feedback was minimally edited for brevity and clarity.

Comments regarding applications seeking authorization for use of GMO-IMPs:

- *"Our experience shows that GMO submissions continue to be challenging. While CTAs have a defined timeline for approval under the CTR, with a coordinated procedure among Concerned Member States, there is still variety in timelines and assessment procedures under two different Directives as legal basis for GMO authorisations across countries that can extend beyond this timeline."*

- *"Specific national requirements, including but not limited to requirements for medicinal products for human use containing or consisting of GMOs, mean that not all EU countries can be included within an initial CTA. For specific EU Member States with particularly burdensome requirements, such as those for Germany*, it is our strategy to add these countries in subsequent applications. However, additional Member State applications create additional complexity. For example, any urgent changes to be made to an initial application would force sponsors to withdraw the additional Member State application. This could result in patients in those additional countries having to wait longer to access innovative medicines."*

    - *It is noted that this experience is not shared by all survey respondents, when, as per question 6, Germany had been identified as a country that would be prioritized for GMO applications, having clear requirements.

- Further than previously stated for these two countries, it was reported that for *"Poland and Czechia, the submission procedure for an GMO application was also unclear"*. Presently, GMO submission documents should not be included with CTAs to be submitted via the Clinical Trials Information System (CTIS) portal, under the CTR. *"However, Poland and Czechia indicated that they may require Contained Use notification documents via a Request for Information (RFI) process. Both countries indicated that such a procedure will be required prior to submission of the CTR (via CTIS) since the EU CTR may not be aligned with their national GMO legislation."*

- *"In one case, Czechia could not be included within our initial CTA submission under the CTR, since authorisation of the application for use of a GMO is required, prior to acceptance of a CTA for a GMO-IMP."*

- *"For one study, GMO submissions were required in 12 countries even though the specific GMO-IMP was identical to that used in a prior clinical trial conducted in the EU for the same indication. GMO submissions were previously made in 7 of the 12 countries (Austria, Belgium, France, Germany, Italy, Norway, and Spain)."*

- *"EU Member State GMO regulatory requirements are difficult to elucidate from national competent authority websites. There is an EC online repository of national GMO regulatory requirements for IMPs (for EU and European Economic Area countries) [25]. However, many summaries date from 2017 and it is unclear whether they are maintained. Evaluation procedures and timelines are not clearly defined and requirements were not recently updated. The contacts for local agencies responsible for the GMO package evaluation are difficult to identify."*

- *"GMO Procedures and timelines are not harmonised across the EU. Currently, each EU Member State requests different submission documents, some of which are required to be in the local language. As an example, for AAV-based IMPs, some countries apply the directive for DR, whereas others apply the directive for the Contained Use procedure. Determining which procedure should be used is not clearly described by all local authorities. It is time-consuming*

and resource-intensive to prepare national submission packages to seek authorisation for approval to use the GMO-IMP, where the long review timelines may also delay start to the clinical study."

- The burdensome nature of work required to populate an EU GMO common application form or a specific ERA (as defined by Annex II of Directive 2001/18/EC) was repeated by another survey respondent.

- *"We had positive experiences when submitting GMO applications for products with marketing authorisation in the following countries: Germany, France, Netherlands, Portugal, Spain, and Sweden. We utilised the Frequently Asked Questions Guidance document "Medicinal products for human use containing or consisting of GMOs: Interplay between the EU legislation on medicinal products and GMOs" guidance (version 3.0 dates October, 2019 [22]). This greatly simplified the GMO process. However, although Austria had endorsed this guidance, the feedback from the Ministry of Health was that they had not utilised it frequently. This led to several requests for information."*

- *"The national GMO procedures are similar to those prior to January 31, 2022 with some level of observed improvement in some countries. However, there is still a lack of clarity at the national level regarding Member State review procedures and associated timelines."*

- *"Together with country affiliates, it was very time consuming to ascertain whether authorisation was required (or not) for use of medicinal products consisting of or containing GMOs, due to lack of harmonisation (across EU Member States). The Frequently Asked Questions Guidance document states that "The GMO framework does not apply to medicinal products that have been granted a marketing authorisation"* [per Article 12 of Directive 2001/18/EC and Article 3(3) of Directive 2009/41/EC] and *"Any use of the medicinal product in accordance with the summary of product characteristics ("SmPC") is therefore exempted from the GMO framework" [22]. However, for a couple of countries, GMO submissions were required for marketed products, in accordance with the SmPC. For example, in Czechia, the local Biosafety officer requested a GMO application."*

- *"EU GMO assessment practices stand in stark contrast to other regions that have GMO assessment requirements but where a waiver can be obtained for low-risk products (e.g., Australia) or where a single assessment can be performed on a per product basis, rather than a per trial basis (e.g., Canada)."*

- *"There is major inconsistency from one EU Member States to another, where some align their evaluation to the EU CTR CTA application evaluation (for example, Germany) and others have a completely separate evaluation, which might lead to updates being required in clinical trial documentation due to GMO questions and/or CTR questions, etc."*

- *"In addition to the survey responses in Question 7, during assessment of the GMO application by the Belgian GMO competent authorities, a new request was raised, asking for creation of additional patient materials specific to handling of the GMO medicinal product. This was an entirely new document. Since the new document is patient facing material, it also needed to be submitted under Part II of the EU CTR submission to be approved for implementation. However the EU CTR review had already commenced. There are system limitations and submission rules with the EU CTR, that is, it is only permissible to respond to queries raised and that ad hoc submissions are not permitted. This necessitated a special request to the Belgian*

Ethics Committee to raise this as a query, in order to open the EU CTR portal to permit submission of the patient facing material. Whilst it was possible at that time, it cannot be guaranteed that such a request will always be successful. Therefore, communication across competent authorities may be beneficial to align on queries raised during review of the GMO application and CTA."

Comments specific to CTAs submitted under the CTR:

- *"Submission of a CTA, under the CTR, via CTIS, was difficult to navigate during our initial submission in 2022. Due to major technical glitches, CTIS did not allow EU Member States to submit RFI queries to the sponsor. This resulted in our need to withdraw the application and to then reapply, causing major delays to the programme (>6 months)."*

- *"A lack of ability to interact directly with the national health authorities has also been a cause for concern. Previously,* [under the Clinical Trials Directive [3,4]] *requested information could be provided to national agencies via email when needed. This option is no longer available."*

- *"A challenging aspect of the new process* [under the CTR] *is the lack of email notifications. For example, when there are notifications or RFIs. This has resulted in the Sponsor needing to log into CTIS every day to ensure that important notifications are not missed and can be addressed promptly and within mandated response times. This daily requirement is quite burdensome. The Sponsor is also not notified or provided with a reason when the evaluation timetable is updated."*

- *"Due to the increased availability of clinical trial materials to the public and increased transparency* [under the CTR] *there is an increased burden of providing redacted documentation for review. It has not been clear to date which documents are considered public facing versus confidential and at which stage of review these documents are necessary. We have provided redacted documentation in the initial application and have received conflicting feedback from the Member States regarding the appropriateness of these documents."*

- As detailed further in the section below, transparency rules for CTIS were revised after the window had closed for this survey.

Summary of industry comments regarding submission of a GMO application and a CTA for an GMO-IMP since January 31, 2022 (per Question 11)

Time- and resource-intensive applications seeking authorizations for use of GMO-IMPs across the EU continue to represent a significant and challenging hurdle for developers.

EU Member State GMO competent authorities have applied differing interpretations of the European Commission Directive for Deliberate Release of GMOs (2001/18/EC; DR [7]) and the Directive on the Contained Use of GMOs (2009/41/EC; CU [9]) that both serve as the legal basis of the GMO framework. As reported previously [8] and captured above through recent survey feedback, there are a variety of GMO competent authority procedures and differing assessment timeframes.

The sequencing of GMO applications and CTAs also differ across EC Member States. There was positive feedback on how the Frequently Asked Questions Guidance document [22] clarified sequencing of GMO and CTAs. However, industry report delayed initiation of clinical trials in Czechia and Poland and how these countries are then not selected for inclusion within initial CTAs.

Despite the positive steps for harmonized forms and streamlined procedures, the benefits of a single CTA submission under

the CTR are diminished, due to different national GMO procedural and documentation requirements, and a lack of formal alignment of timelines between CTA and GMO procedures.

After a public consultation was opened in May 2023 [26], transparency rules for CTIS were revised after the survey window had closed [27]. Survey respondents had noted a lack of clarity with regard to confidentiality and differing requirements across Member State health authorities. The EMA is preparing to roll out simplified transparency rules for CTIS mid-2024. Sponsors with trial applications in CTIS can switch to the new simplified transparency rules when an opportunity arises to make changes to the study, e.g., substantial modifications or the addition of a new Concerned Member State [28].

Survey respondent feedback also refer to more pragmatic approaches taken by Australian and Canadian authorities to control any risk to the environment, including where a waiver can be obtained for low-risk products (in Australia).

RECENT CLARIFICATIONS FOR USE OF A GMO-IMP IN A CLINICAL TRIAL

In 2021 the French law simplified the process for GMO-IMPs [29]. As of January 1, 2022, the responsibility for declaring the use of a GMO that poses zero or negligible risk falls on the Sponsor, rather than on the individual sites involved in a clinical trial. The Sponsor makes a single submission for the trial, citing the involved clinical sites. As of June 1, 2022, Sponsors were able to send declarations via an online portal to the National Agency for Medicines and Health Products (ANSM) instead of The Ministry of Higher Education, Research and Innovation (MESRI).

In October 2023, the Federal Agency for Medicines and Health Products (FAMHP) of Belgium (and Sciensano) updated "Belgian regulatory guidance on the use of genetically modified organisms in a clinical trial" [30]. This included the requirement that applies to all Member States that since October 1, 2022, Sponsors are required to submit Summary Notification Information Format (SNIF) documents for new clinical trial applications through the E-Submission Food Chain (ESFC [31,32]) platform, although there is no current mechanism for a single notification to the ESFC of multinational trials.

As noted by the FAMHP [33], an ERA (within a biosafety dossier) cannot be submitted via CTIS and, for the FAMHP, must be submitted in parallel (to a CTA) via the Common European Submission Portal to the FAMHP Research and Development Division. For greater predictability to timing of authorization of both CTA and ERA (in the biosafety dossier) the FAHMP Research and Development Division seeks alignment with regard to timing of decision, under the condition that both the CTA and the biosafety dossier are submitted at the same time, and that the Sponsors responds to any validation and assessment questions within the same timelines dictated by the CTR.

For deliberate release of a GMO-IMP, in parallel to the evaluation of the biosafety assessment by The (Belgian) Biosafety and Biotechnology service [34], a 30-day public consultation of the dossier must be allowed. The applicable Belgian legislation (Royal Decree of February 21, 2005 [35]) states that this public consultation has to take place in parallel to the biosafety assessment of the IMP by the GMO authorities and should be finished by the time these authorities transmit their advice to the competent authorities for the evaluation of the clinical trial (the FAMHP).

REFORM OF THE EU PHARMACEUTICAL LEGISLATION, AS APPLICABLE TO GMO INVESTIGATIONAL MEDICINES

On April 26, 2023 the European Commission adopted a proposal for a new Directive and

a new Regulation, which revise and replace the existing general EU pharmaceutical legislation [36]. The draft proposed Regulation [37] includes a reform to the regulation of medicinal products that contain or consist of GMOs. Within the proposal document, it is acknowledged that, *"It is particularly difficult to conduct multi-centre clinical trials with investigational medicinal products that contain or consist of GMOs involving several Member States."*

The following articles are relevant to the proposals applicable to GMOs: Regulation Recital 53, 54; Article 6(6); Articles 7–9 (MAA); Article 177 (CTA).

In the proposal, Article 177, and recitals 53 and 145 through to 152, amend the CTR. Identified key points of interest to developers of GMO-IMPs are bulleted below.

The proposed Regulation Article 177 amends Regulation 536/2014 (CTR): Article 5(a): 'Environmental risk assessment (ERA) for investigational medicinal products for human use containing or consisting of genetically modified organisms':

- The EC proposed that it is necessary to subject GMO medicinal products to an ERA procedure similar to that for the Deliberate Release (DR) Directive (2001/18/EC). The ERA procedure is to be conducted in parallel with the evaluation, under a single European Union procedure, of the quality, safety and efficacy of the product concerned (through an Investigational Medicinal Product Dossier [IMPD]);
- Sponsors shall submit an ERA (for a GMO-IMP) via the EU portal, CTIS;
- The EMA Committee for Medicinal Products for Human Use (CHMP) shall assess the ERA in the form of a scientific opinion. The CHMP shall submit its opinion to the competent authority of the Reporting Member State within 45 days from the validation date:
  - Where appropriate, the opinion shall include risk mitigation measures;
  - The sponsor shall provide evidence to the Reporting Member State and the Concerned Member States that these measures will be implemented;
  - The CHMP may request, with justified reasons, via CTIS, additional information from the sponsor;
  - The EMA may extend the assessment period by a maximum of 31 days.
- ERA principles are to be set out in Annex II to the Directive for DR. Scientific guidelines are to be developed by the EMA in coordination with competent authorities of the Member States. Any future changes to Annex II of 2001/18/EC will affect the ERA for clinical trials.

Other proposals were included within the Regulation and are of considerable importance, as they apply to patient access to GMO medicines, without the need for an ERA, under specific circumstances:

- The revised Directive relating to Medicinal Products for Human Use (2001/83/EC [38]) will temporarily allow for the use and supply of unauthorized medicinal products for urgent and exceptional public health reasons, without an ERA.
- Member States will now be allowed to make a medicinal product available for Compassionate Use [39] without the prerequisite for an ERA, prior to its marketing authorization.

EFPIA assessment

Harmonization via a centralized assessment of an ERA and GMO documentation that follow defined timelines, consistent

with those of the CTR, represents a vast improvement to the current fragmented requirements. Such a centralized assessment of GMO-IMPs will still involve environmental experts from national GMO competent authorities but will remove the current divergence(s) between the Member States with respect to the determination of applicable GMO data requirements and documentation.

During assessment of the proposals, some gaps were identified regarding our current understanding of future processes, when submitting a centralized ERA for a GMO-IMP via CTIS:

- The EU CTIS will be adapted to accept GMO submissions for IMPs. There is a current lack of clarity for GMO-specific national Member State clinical site-specific documentation and if such documents are to be provided via CTIS as a part I document (or if independent to part I);
- Some areas will need to be addressed in detail regarding delegated acts or guidance. For example, Company Confidential Information, etc.;
- The procedure for submission and harmonized assessment of an ERA for GMO-IMPs to be specified by the EC in a delegated act. Clarity on the procedure, including a comprehensive list of required GMO submission documents are, in due course, anticipated, per the proposed procedure based on the principles of the Directive for DR (2001/18/EC);
- Paragraph 8 of Article 5a refers to *"the content of the ERA taking into account the common application forms and Good Practice Documents for genetically modified human cells and for adeno-associated viral vectors that were published by the Agency"*. The adoption of revised CAFs reflecting a risk-based approach through the centralized assessment would be welcomed by industry.

REFORM OF THE EU PHARMACEUTICAL LEGISLATION, PER EC PROPOSALS FOR ERAS AT THE TIME OF MARKETING AUTHORIZATION APPLICATION

Articles 7 to 9 of the proposed regulation are specific to the procedure and content of an ERA of proposed regulation on the procedure and content of the ERA for medicinal products containing or consisting of GMOs as part of the marketing authorization application (MAA): The MAA shall be accompanied by an ERA identifying and evaluating potential adverse effects of the GMO on human health and the environment. As per that for GMO-IMPs, the ERA for a MAA shall be conducted in accordance with principles set out in Annex II to the Deliberate Release Directive 2001/18/EC. Information pertaining to the environmental risk shall appear as an appendix to Module 1.

Assessment

The EC proposed requirements at the time of MAA is in accordance with the current requirements at time of MAA in accordance with the 2006 EMA 'Guideline on Environmental Risk Assessment for Medicinal Products Consisting of or Containing Genetically Modified Organisms' [40]; Q 3.4.3 of EMA Pre-authorisation guidance [41]; and Directive 2001/83/EC Annex I, Part I, 1.6 (*"Where applicable, applications for marketing authoristions shall include a risk assessment overview evaluating possible risks to the environment due to the use and/or disposal of the medicinal product and make proposals for appropriate labelling provisions"* [38]).

It is also considered positive to how the CHMP will assess GMO submissions, for both clinical trials and MAAs. Consultation with national Member State GMP experts

will or may only be required in cases of first-in-class products, or when new questions are raised during assessment of the GMO medicinal product. Even first-in-class GMO medicines are often based on well-known platforms, where environmental risks will have already been assessed parallel to, or part of a CTA.

MAY 16, 2023 EMA COMMITTEE FOR ADVANCED THERAPEUTICS (CAT) STAKEHOLDER MEETING

In the May 16, 2023 EMA CAT Stakeholder Meeting [42], Industry provided information regarding 'Experiences and Issues with ATMPs Consisting of GMOs'. Dr Lina Koufokotsiou, representing the Commission, clarified to how, as proposed in the revised General Pharmaceutical Legislation (GPL), national GMO submissions for clinical trials would no longer be needed.

Another important clarification was that the Contained Use Directive (2009/41/EC) will no longer be applicable, and there will be no requirement for national notification by the Sponsor.

There was further clarification that, despite there being reference to Annex II of the Deliberate Release Directive within the proposal text, that the future ERA will be tailored to the requirements for investigational medicinal products (and not plants).

However, it will be important that the above understanding is clarified in the implementation documents, following the adoption of the GPL.

OCTOBER 3, 2023: ENVI RAPPORTEUR DRAFT REPORT FOR THE REGULATION

Tiemo Wölken, Member of European Parliament (MEP, Germany) representing the Socialists and Democrats group, is the rapporteur who chaperones the Regulation through negotiations. [Pernille Weiss, MEP (Denmark), representing the European People's Party, leads the discussion on the proposed Directive]. On October 3, 2023, MEP Wölken released his amendments for parliamentary negotiation in the European Parliament Committee on the Environment, Public Health and Food Safety (ENVI) rapporteur draft report for the Regulation [43]. Whilst MEP Wölken's draft negotiation report for the regulation did not contain amendments to the Commission's proposals as they apply to the CTR and Article 177, there were two proposed amendments to Articles 7–9 of the proposed regulation on the procedure and content of the ERA for medicinal products containing or consisting of GMOs as part of the MAA:

The first proposed amendment regards the content of an MAA for an ERA, the following was proposed, as inserted into Article 8 (b): *"[…] for the purpose of this point, 'hazards for human health' includes the risks to the health of human beings other than the treated patient as the risk to the treated patient shall be assessed as part of the benefit–risk assessment of the medicinal product;"*

The second proposed amendment (to article 9, paragraph 2) proposes that, for first-in-class medicinal products or when a novel question is raised during the assessment of the submitted ERA, the CHMP, or the rapporteur, shall carry out necessary consultations with bodies Member States set up in accordance with Directive 2001/18/EC. Furthermore, *"they **shall** also consult with relevant Union bodies, **inter alia the European Environment Agency**"*. This represents greater regulatory oversight of novel GMO-IMPs.

In an explanatory statement at the end of the draft report, MEP Wölken states his, *"positive* [view] *regarding the enhanced provisions on the Environmental Risk Assessment proposed by the Commission and the proposed restructuring of the European Medicines Agency that will facilitate streamlined procedures and accelerated marketing authorisations"*.

It is also stated how, *"the rapporteur would oppose derogations from the requirements set*

out in both the Regulation and the Directive, particularly with regards to the Environmental Risk Assessment".

December 4, 2023, MEPs of ENVI tabled amendments on the GPL: proposal for future risk-based approach to GMO ERA requirements

On December 4, 2023, MEPs of ENVI tabled their amendments to the GPL Regulation and Directive. Twelve amendments were specific to GMO medicinal products. The majority of the proposed amendments were minor improvements, or neutral minor edits, to that proposed by the Commission in April 2023.

There were three amendments relating to risk-based approaches that would substantially improve upon the EC proposals from April last year. This includes the proposed amendment tabled by MEP Anders Vistisen, on behalf of the Identity and Democracy Group [44]. The amendment, numbered 294, along with amendments 1896 and 1897, builds upon the initial Commission proposal by including future consideration of a risk-based approach to GMO ERA requirements:

Amendment 294 foresees future adaptation of the Regulation and to the revised Directive on Medicinal Products for Human Use (2001/83/EC) *"to lay down specific rules for these categories of medicines on the basis on an evaluation"*. This is to ensure that the proposed legislation *"does not impede the development and approval of novel medicines containing and consisting of GMOs"* [38].

Amendment 1896 includes inserted text "[…] *where the sponsor can demonstrate the absence or low environmental risks from a GMO-IMP, the sponsor shall then not be required to conduct the assessment prior to commencement of a clinical trial."*

Following on, amendment 1897 includes inserted text regarding how a delegated act shall include *"a list of well-characterised investigational medicinal products containing or consisting of GMOs that do not survive in the environment."*

Such a risk-based approach and non-requirement for an ERA for well-characterized GMOs that pose negligible risk to the environment has been previously advocated for by EFPIA and would be welcomed broadly by industry.

Amendment 298 (MEP Tilly Metz, Greens /European Free Alliance) is specific to the EC proposed non-requirement for an ERA for a GMO-IMP under Compassionate Use. The proposed inserted text into Recital 54 is shown in bold: *"Member States should implement appropriate measures to* ***prevent*** *foreseeable negative environmental impacts resulting from the intended or unintended release of the medicinal products containing or consisting of GMOs into the environment* ***and agree on an appropriate timeline for the delivery of the environmental risk data****"*. In this context, the provision of what data to be provided is queried. Non-clinical biodistribution and shedding data may not be available, e.g., for a novel or variant viral vector, with possible different tropism to that reported in literature. In this context, it is proposed that "data" be replaced with "assessment". Future CHMP guidance could assist best practice within the Compassionate Use scenario, so that ERA requirements do not delay provision of the medicine to a patient with an unmet medical need.

Amendment 779 (MEP Stanislav Polčák, European People's Party) is specific to the EC-proposed Temporary Emergency Marketing Authorisation (TEMA) prior to provision of environmental risk assessment. TEMA is intended for medicinal products, *"for the treatment, prevention or medical diagnosis of a serious or life-threatening disease or condition which are directly related to the public health emergency, prior to the submission of the complete quality, non-clinical, clinical data and environmental data and information"*. The inserted amendment text (shown in bold) adds that TEMA only be granted, ***"if the best available scientific information***

indicates that there is no risk of serious or irreversible harm to the environment, animals or human health […]". This amendment, although possibly unclear, still asks for the *"best scientific information"*. And if that information is not within an ERA, then clarification could be required to determine what level of documentation would suffice.

SUMMARY OF KEY PROPOSALS BY THE EUROPEAN COMMISSION AND AMENDMENTS BY THE EUROPEAN PARLIAMENT

- Through the European Commission's reform of the GPL, instead of needing to submit GMO dossiers to Member State GMO Competent Authorities for each country where it is intended to perform a clinical trial, the EC proposes harmonization through a centralized, single Union procedure, to be conducted in parallel to a CTA;
- The EMA CHMP shall assess the GMO submission (that includes an ERA) in the form of a scientific opinion, for both clinical trials and marketing authorization applications. The ERA will be tailored to the requirements for investigational medicinal products (instead of GMO plants);
- The GMO requirements will follow principles similar to that of the Deliberate Release Directive (2001/18/EC);
- The Contained Use Directive (2009/41/EC) will no longer be applicable and there will be no national notification requirements under this Directive;
- MEPs have tabled amendments to the proposals that include a risk-based approach. This includes the non-requirement for an ERA of investigational medicines with demonstrated low environmental risk, prior to a clinical trial.

OUTLOOK

Currently, there is a high level of alignment within the EC and European Parliament (EP) regarding the industry's position on appropriate regulation of medicinal products that contain or consist of GMOs. There is minor misalignment with the Green Party, without any identified major opposition.

On April 10, 2024, the EP held the plenary vote on the GPL. MEPs voted in favor of the EP's proposal to reform the pharmaceutical legislation for the first time in 20 years [45]. The file on the GPL will be followed up by the new Parliament after the June 6–9 European elections, and the file moves to the Council of the EU which represents the 27 Member States. At the end of the full process, both the EP and European Council will adopt the legislation, which we are a few years away from, with a projected implementation timeframe of 2028.

CONCLUSION

The industry survey shows that under the CTR, national authorization for use of GMO-IMPs continue to cause considerable concern across industry, due to the current heterogeneity in assessment procedures, requirements, and timeframes across Member State GMO Competent Authorities and between them and those authorities in charge of clinical trial applications assessments. Member State GMO regulatory requirements are difficult to elucidate from available information that includes national authority websites. In addition to the time and resources needed to prepare ERAs, CAFs, SNIFs, and other national forms, the survey highlights how, for nearly every GMO authority, re-submissions are required for GMO-IMPs identical to that used in a previous clinical trial for the same indication. However, in The Netherlands and Italy, simpler and expedited procedures exist for notification of intended use of a previously reviewed and approved GMO. Other regions have been considered by Industry to

be more attractive than the EU to undertake clinical trials for GMO-IMPs, when waivers can be obtained for low-risk products (e.g., Australia) or where a single assessment can be performed on a per product basis, rather than a per trial basis (e.g., Canada).

As per the EC Impact assessment report and executive summary accompanying the revision of the GPL, published April 2023 [46], *"streamlining the GMO assessment in the authorisation of clinical trials that involve investigational medicines with a GMO component" "adds clarity and predictability to the regulatory system and the legal pathway". "These measures should promote innovation and attract investment to the EU".* Until a final version of the GPL is implemented in 2028, the EU may lose further ground, with sponsors avoiding the region to undertake clinical trials with GMO-IMPs, further reducing patient access for rare indications in the EU.

Whilst it is acknowledged that this report provides context to the current part of the process of revision of the European GPL as it applies to GMO medicines, EFPIA supports and would welcome the EC-proposed harmonization via an EU-wide centralized assessment of ERAs. Furthermore, industry would continue to advocate for such a risk-based approach and non-requirement for an ERA for well-characterized GMOs (as per the proposed amendment [#1896]) to the Regulation.

REFERENCES

1. *European Union*. Regulation (EU) No 536/2014 of the European Parliament and of the Council of April 16, 2014 on Clinical Trials On Medicinal Products For Human Use, And Repealing Directive 2001/20/EC.

2. European Medicines Agency Clinical Trials Regulation.

3. European Union. Directive 2001/20/EC of the European Parliament and of the Council of April 4, 2001.

4. *European Commission*. Clinical trials—Directive 2001/20/EC.

5. *European Union*. Clinical Trials Regulation (EU) No 536/2014 in Practice. The Rules Governing Medicinal products in the European Union 2024.

6. *European Medicines Agency*. Clinical Trials' Transition To New EU System—1 Year Left 2024.

7. Directive 2001/18/EC of the European Parliament and of the Council of March 12, 2001 on the Deliberate Release into the Environment of Genetically Modified Organisms and Repealing Council Directive 90/220/EEC.

8. Lambot N, Awigena-Cook, Reimer T, *et al*. Clinical trials with investigational medicinal products consisting of or containing genetically modified organisms: implementation of Clinical Trials Regulation EU 536/2014. *Cell Gene Ther. Insights* 2021; 7(9), 1093–106.

9. Directive 2009/41/EC of the European Parliament and of the Council of May 6, 2009 on the contained use of genetically modified micro-organisms.

10. *European Medicines Agency*. Guideline on the Scientific Requirements for the Environmental Risk Assessment of Gene Therapy Medicinal Products. May 30, 2008.

11. Alliance for Regenerative Medicine (ARM), European Federation of Pharmaceutical Industries and Associations (EFPIA), European Association for Bioindustries (EuropaBio). Call for more effective regulation of clinical trials with advanced therapy medicinal products consisting of or containing genetically modified organisms in the European Union. *Hum. Gene Ther*. 2021; 32, 19–20.

12. *European Union*. Common Application Form For Clinical Research With Genetically Modified Human Cells. Version 5; 2021.

13. *European Union*. Common Application Form For Gene Therapies That Contain Or Consist Of AAV. Version 3; 2022.

14. *European Union*. Common Application Form For Form For Viral Vectors Contained In Investigational Medicinal Products For Human Use. Version 3; 2022.

15. *European Union*. Good Practice on the Assessment of GMO-Related Aspects in the Context of Clinical Trials with Human Cells Genetically Modified. Version 5; 2021.

16. *European Union*. Good Practice on the Assessment of GMO Related Aspects in the Context of Clinical Trials with AAV Clinical Vectors. Version 3; 2022.

17. Alliance for Regenerative Medicine. Regenerative medicine: the pipeline momentum builds; 2022.

18. *US Department of Health and Human Services Food and Drug Administration Center for Biologics Evaluation and Research*. Determining the Need for and Content of Environmental Assessments for Gene Therapies, Vectored Vaccines, and Related Recombinant Viral or Microbial Products. Guidance for Industry 2015.

19. *US Food and Drug Administration*. Code of Federal Regulations Title 21, Part 25. Environmental Impact Considerations. Subpart C—Categorical Exclusions. Sec. 25.31 Human Drugs and Biologics 2020.

20. *Government of Canada*. What We Heard: Stakeholder pre-consultation on the review of Part 6 of the Canadian Environmental Protection Act, 1999 (CEPA) and the New Substances Notification Regulations (Organisms) 2023.

21. *Australian Government Department of Health and Aged Care*. National Gene Technology Scheme, Consultation Regulation Impact Statement (CRIS) Published Responses.

22. *European Commission*. Medical Products for Human Use Containing or Consisting of GMOs: Interplay between the EU Legislation on Medical Products and GMOs, Frequently Asked Questions. Version 3; 2019.

23. *Commissie Genetische Modificatie (COGEM)*. Advice Following the European Consultation on the Authorisation Process of GMOs in Medical Applications. COGEM 20.08.2021; CGM/210820-01.

24. American Society of Gene and Cell Therapy. Cell and Gene Therapy dashboard. Citeline; 2023.

25. *European Commission*. Genetically Modified Organism (GMO) Aspects for Investigational Medicinal Products.

26. *European Medicines Agency*. Review of Transparency Rules for the EU Clinical Trials Information System (CTIS) 2023.

27. *European Medicines Agency*. Revised Transparency Rules for the EU Clinical Trials Information System (CTIS) 2023.

28. Pink Sheet. Navigating The EU's Revised Clinical Trial Disclosure Rules 2024.

29. ANSM. Declaration/autorisation d'utilisation confinée ou de dissémination volontaire des médicaments contenant ou composés d'organismes génétiquement modifié.

30. *Federal Agency for Medicines and Health Products and Sciensano*. Belgian Regulatory Guidance on the Use of Genetically Modified Organisms in a Clinical Trial 2023.

31. European Commission. E-Submission Food Chain.

32. European Commission. Food safety: training and support.

33. *Federal Agency for Medicines and Health Products.* Clinical Trials with Genetically Modified Medicinal Products 2023.

34. Belgian Biosafety and Biotechnology service.

35. Belgian Royal Decree of February 21, 2005.

36. *European Commission.* Reform of the EU Pharmaceutical Legislation 2023.

37. *European Commission.* Regulation of the European Parliament and of the Council Laying Down Union Procedures for the Authorisation and Supervision of Medicinal Products for Human Use and Establishing Rules Governing the European Medicines Agency, Amending Regulation (EC) No 1394/2007 and Regulation (EU) No 536/2014 and repealing Regulation (EC) No 726/2004, Regulation (EC) No 141/2000 and Regulation (EC) No 1901/2006.

38. Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community Code Relating to Medicinal Products for Human Use.

39. European Medicines Agency. Compassionate use.

40. *European Medicines Agency.* Guideline on Environmental Risk Assessment for Medicinal Products Consisting of or Containing Genetically Modified Organisms. Committee for Medicinal Products for Human Use 2006.

41. *European Medicines Agency.* Pre-Authorisation Guidance 2024.

42. European Medicines Agency. Committee for Advanced Therapeutics Stakeholder Meeting, May 16, 2023.

43. European Parliament. Draft Report on the Proposal for a Regulation of the European Parliament and of the Council Laying Down Union Procedures for the Authorisation and Supervision of Medicinal Products for Human Use and Establishing Rules Governing the European Medicines Agency, amending Regulation (EC) No 1394/2007 and Regulation (EU) No 536/2014 and repealing Regulation (EC) No 726/2004, Regulation (EC) No 141/2000 and Regulation (EC) No 1901/2006 (COM[2023]0193—C9-0144/2023—2023/0131[COD]). Committee on the Environment, Public Health and Food Safety. October 3, 2023.

44. European Parliament Identity and Democracy Group.

45. European Parliament news. Parliament adopts its position on EU pharmaceutical reform (April 10, 2024).

46. *European Commission.* Impact Assessment Report and Executive Summary Accompanying the Revision of the GPL 2024.

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CELL & GENE THERAPY INSIGHTS

GENE THERAPY ANALYTICS AND CMC

SPOTLIGHT

INNOVATOR INSIGHT

Quantitative analysis of lipids and nucleic acids in lipid nanoparticles using monolithic column

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Lipid nanoparticles, a promising platform for drug delivery, effectively encapsulating and protecting nucleic acids. Comprising cationic or ionizable lipids, helper lipids, and PEGylated lipids, LNPs facilitate cellular uptake and control of payload release. The composition of lipids is crucial for optimizing LNP formulations, necessitating robust analytical methods. This study presents the development of a reverse phase liquid chromatography method to separate and quantify lipids and nucleic acid in LNP formulations. The method utilizes a PATfix® analytical system equipped with an evaporative light scattering detector and a monolith CIMac™ C4 HLD chromatographic column. The method demonstrated efficient lipid separation and detection, with validation following international chromatography handbook guidelines, highlighting its sensitivity, linearity, precision, and accuracy. Furthermore, the method's suitability for quantitative analysis was verified by assessing lipid ratios in various LNP formulations, confirming its applicability for monitoring lipid composition throughout the LNPs' manufacturing process.

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Lipid nanoparticles (LNPs) have emerged as versatile drug delivery systems capable of encapsulating various payloads, including different nucleic acids [1,2]. LNPs offer numerous advantages, such as protection of the payload from degradation, enhanced cellular uptake, and controlled release, making them promising candidates for therapeutic applications [3].

Lipid nanoparticles (LNPs) typically comprise cationic or ionizable lipids, helper lipids, and PEGylated lipids [4–6]. Cationic or ionizable lipids play a crucial role in enhancing the binding and transfection efficiency of RNA. Cationic lipids, such as DOTAP, possess a permanently charged headgroup, while ionizable lipids, such as SM-102, acquire charge in lower pH environments (around a pH of 6–7) and remain uncharged at physiological pH levels (around 7.4). The positively charged headgroups of these lipids interact electrostatically with the negatively charged phosphate backbone of RNA, facilitating better RNA-LNP association. The manufacturing of LNPs typically occurs under acidic conditions (pH 4), often implemented in microfluidic systems. Additionally, ionizable lipids offer the advantage of improving transfection efficiency, partly due to enhanced endosomal escape. Helper lipids, such as cholesterol, DSPC, or DOPC, contribute to the stability and rigidity of LNPs, and they also influence cellular processes such as endocytosis. The incorporation of PEGylated lipids into LNPs affects a number of physiological processes, including prolonged blood circulation, half-life, and *in vivo* distribution. Furthermore, these lipids influence the characteristics of LNPs, such as size, encapsulation efficiency, and aggregation.

The advantageous characteristics of lipids and their compositions in the formulation of RNA necessitate the utilization of suitable analytical methods throughout the drug development process. It is therefore essential that these methods enable the quantification of individual components and facilitate stability studies. To devise an analytical approach for lipid quantification, various chromatographic techniques can be explored. Among these, high-performance liquid chromatography (HPLC) stands out as a widely embraced method.

Different HPLC modes can be employed for analysis of lipids; normal phase liquid chromatography (NPLC) [7–9], and most commonly used reverse phase liquid chromatography (RPLC) [10]. In RPLC, a non-polar stationary phase (typically C18, C8) is used, and separation is based on the hydrophobic interactions between the lipid molecules and the stationary phase [11–13]. The choice of detector in HPLC hinges upon the properties of the analytes under examination. Given that most lipids lack chromophores, UV detectors may prove inadequate for analyzing LNPs. Other analytical techniques, such as mass spectrometry (MS) [14–16], refractive index detectors (RID) [17], evaporative light scattering detectors (ELSD) [18], and charged aerosol detector (CAD) [19–21] have been employed for lipid analysis. However, MS is often considered too costly for routine use, and developing a thoroughly validated method presents challenges. RID suffers from inherent limitations, primarily low sensitivity, while CAD struggles with poor signal-to-noise ratios. ELSD is garnering significant interest due to its capability to measure charged particle signals with an electrometer. Moreover, its response is generally independent of the chemical structure of the analyte, with volatility being a more critical factor.

In this study, a reverse phase liquid chromatography (RPLC) method, utilizing a PATfix® chromatographic system equipped with an ELSD and a monolith CIMac™ C4 HLD analytical column, was developed to separate and quantify lipids and nucleic acid in different LNP formulations. The developed analytical method was applied to analyze the lipid composition of LNPs and to compare the lipid components across different LNP formulations. This method allows for a direct injection of LNP formulations into the chromatographic system, obviating the need for dissolution or disassembly of the LNPs.

MATERIALS AND METHODS

Materials

Triethylammonium acetate buffer (TEAA, HPLC grade) and sucrose (≥99.5) were obtained from Sigma-Aldrich (Taufkirchen, Germany), Dulbecco's phosphate buffered saline (PBS) from VWR (Lutterworth, UK) and isopropanol (IPA, LC-MS grade) from Merck (Darmstadt, Germany). Buffers were freshly prepared with LC-MS grade water purchased from Honeywell (Seelze, Germany). mFix4 was obtained from Sartorius BIA Separations (Ajdovščina, Slovenia), cationic lipid LipidBrick® IM21.7c from Polyplus (Illkirch, France), ionizable lipid SM-102 from Biosynth (Bratislava, Slovakia) and cholesterol from Sigma-Aldrich (Taufkirchen, Germany), while lipids DOPC, DSPC and DMG-PEG2k were purchased from Avanti (Alabaster, AL, USA).

LNP sample preparation

Two LNPs with different lipid composition were prepared. Lipids used for cationic lipid LNP were LipidBrick® IM21.7c, DOPC, cholesterol and DMG-PEG2k (molar ratio 33.3:6.7:25.7:1.0), while SM-102, DSPC, cholesterol and DMG-PEG2k (molar ratio 28.9:5.6:26.7:1.0) were used for ionizable lipid LNP. For both LNPs, mRNA mFix4 was used. LNPs were prepared using NanoAssemblr™ Ignite™ system with NxGen™ cartridge (Precision NanoSystems, Vancouver, Canada) and were buffer exchanged to storage buffer (1× PBS, 10% sucrose, pH 7.4) with 30 kDa cut-off Amicon filters (Merck, Darmstadt, Germany).

PATfix analytical system connected to ELSD

Chromatographic experiments were performed using PATfix analytical system, with a quaternary pump, a multiwavelength UV–Vis detector, a column thermostat and 8-port valve (Sartorius BIA Separations). For lipid detection, SEDEX LT-ELSD LC™ (Knauer, Berlin, Germany) was used. Samples were analyzed with 0.1 mL CIMac C4 analytical column (2 mL channel size) (Sartorius BIA Separations). Before analysis, samples were diluted with 10 mM TEAA and IPA (3:1). Injection volume was 500 mL. Sample analysis was monitored using UV detection at 260 and 280 nm. ELSD evaporation temperature was set to 53 °C and the C4 column was kept at 30 °C. For instrument control and data processing, PATfix software (Sartorius BIA Separations) was used.

RESULTS AND DISCUSSION

Lipids and mRNA separation and determination

The analysis of LNPs in a single assay presents a significant challenge due to their complex composition, which typically includes nucleic acid and at least four different lipid species. The diverse polar properties of these components make comprehensive analysis on reverse-phase columns difficult. A challenge arises from the suboptimal interaction between polar nucleic acids and the polar head groups on lipids with reverse-phase columns [18]. Consequently, the employment of an alternative column or the modification of the mobile phase may result in enhanced outcomes. Zhong and colleagues initially proposed an effective approach for the detection and separation of DOTAP using a C18 column, with TFA included in the mobile phase. TFA serves to protect the positively charged headgroups of cationic and ionizable lipids, thereby extending their interaction with the stationary phase.

The objective of this study is to expand the scope of lipid analysis assays to encompass the detection of nucleic acid in LNP formulations. The substitution of TFA with the less aggressive ion-pairing reagent TEAA played a pivotal role in alleviating the issue of excessive and irreversible interaction between mRNA

in LNP formulations and the C4 reverse-phase column. This substitution effectively facilitated the separation of all lipids in the LNP formulation, while promoting efficient binding of the nucleic acid.

Figure 1 illustrates two examples of the separation of four distinct lipids commonly used in LNP formation. The upper section of the figure displays UV signals at 260 nm, while the lower section shows the ELSD signal trace. Due to the absence of chromophores, lipids do not exhibit any response in the UV section of the chromatogram. Instead, their presence is detected solely in the ELSD section. In Figure 1A, an ionizable lipid is employed alongside cholesterol, a phospholipid, and a pegylated lipid. In Figure 1B, a cationic lipid is used in conjunction with cholesterol, a phospholipid, and a pegylated lipid. In both scenarios, separation was achieved using a C4 HLD column with ELSD detection in an increased isopropanol gradient. The method yielded distinct peaks and a baseline separation of all four lipids. The elution order of all four lipids is governed by their hydrophobic nature. The more polar lipids are eluted first, followed by the phospholipid, cholesterol, and then the ionizable/cationic lipid, respectively.

In order to assess the suitability of the method for LNP formulation investigation, LNPs were directly analyzed using this method without any sample pre-treatment, with the exception of dilution with the loading buffer. The resulting chromatograms of LNPs containing ionizable and cationic lipid are shown in Figure 2A & B, respectively.

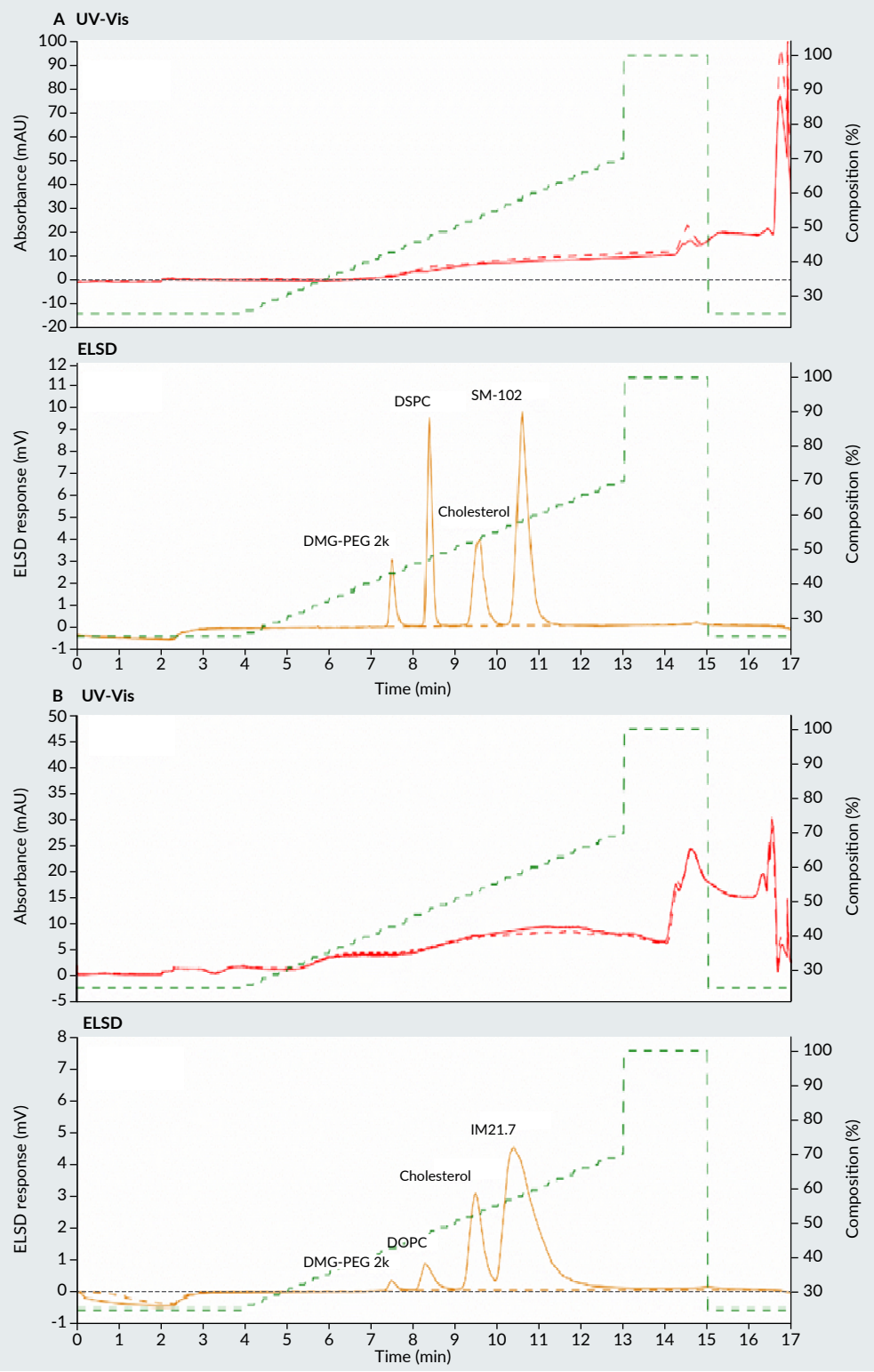
In the case of the ionizable lipid LNP formulation (Figure 2A), it can be demonstrated that all the lipids composing the LNPs are effectively separated. Furthermore, an additional peak is observed in the UV region of the chromatogram, which represents the mRNA. It is crucial to acknowledge that this analytical method is unable to differentiate between naked mRNA and encapsulated mRNA. The LNPs were destroyed on the C4 column during the analysis, resulting in the co-elution of naked and encapsulated mRNA. A comparable observation is made for cationic lipid LNPs (Figure 2B), where all four lipids are distinctly separated in the ELSD portion of the chromatogram. In this instance, the mRNA peak is also observed in the UV spectra, indicating the elution of the mRNA. For cationic lipid LNPs, the mRNA elutes at a higher concentration of isopropanol, indicating greater hydrophobicity of the eluted mRNA. This phenomenon may result from the presence of the permanently charged cationic lipid, which can act as an ion-pairing reagent. The polar, positively charged head of the cationic lipid interacts with the negatively charged phosphate groups on the mRNA, while the non-polar tail of the lipid strongly interacts with the C4 column, thus prolonging the elution of mRNA. This interaction illustrates the reason why the elution of the mRNA occurs simultaneously with the elution of the cationic lipid from the C4 column. Consequently, the ELSD elution peak of the cationic lipid exhibits a different shape compared to the elution profile when mRNA is not present in the sample. In addition to the ability to detect and quantify the lipids, this method also permits the detection and quantification of the total mRNA in LNPs in the same assay.

Method validation

The developed chromatographic method has been validated by evaluating several criteria, including sensitivity (limits of detection and quantification), linearity, precision (repeatability, intermediate repeatability) and accuracy all included in ICH guidance for analytical method validation [22]. The validation results are presented in Table 1. LOD and LOQ for mRNA were determined from signal to noise ratio of 3 and 10, respectively, using UV 260 nm absorbance signal. For LOD and LOQ of lipids, ELSD signal was used instead (Table 1). The linearity of the method was evaluated at six concentration levels for each of the six lipids and mRNA. ELSD signal does not vary linearly as function of the injected mass but in our case follows polynomial

FIGURE 1

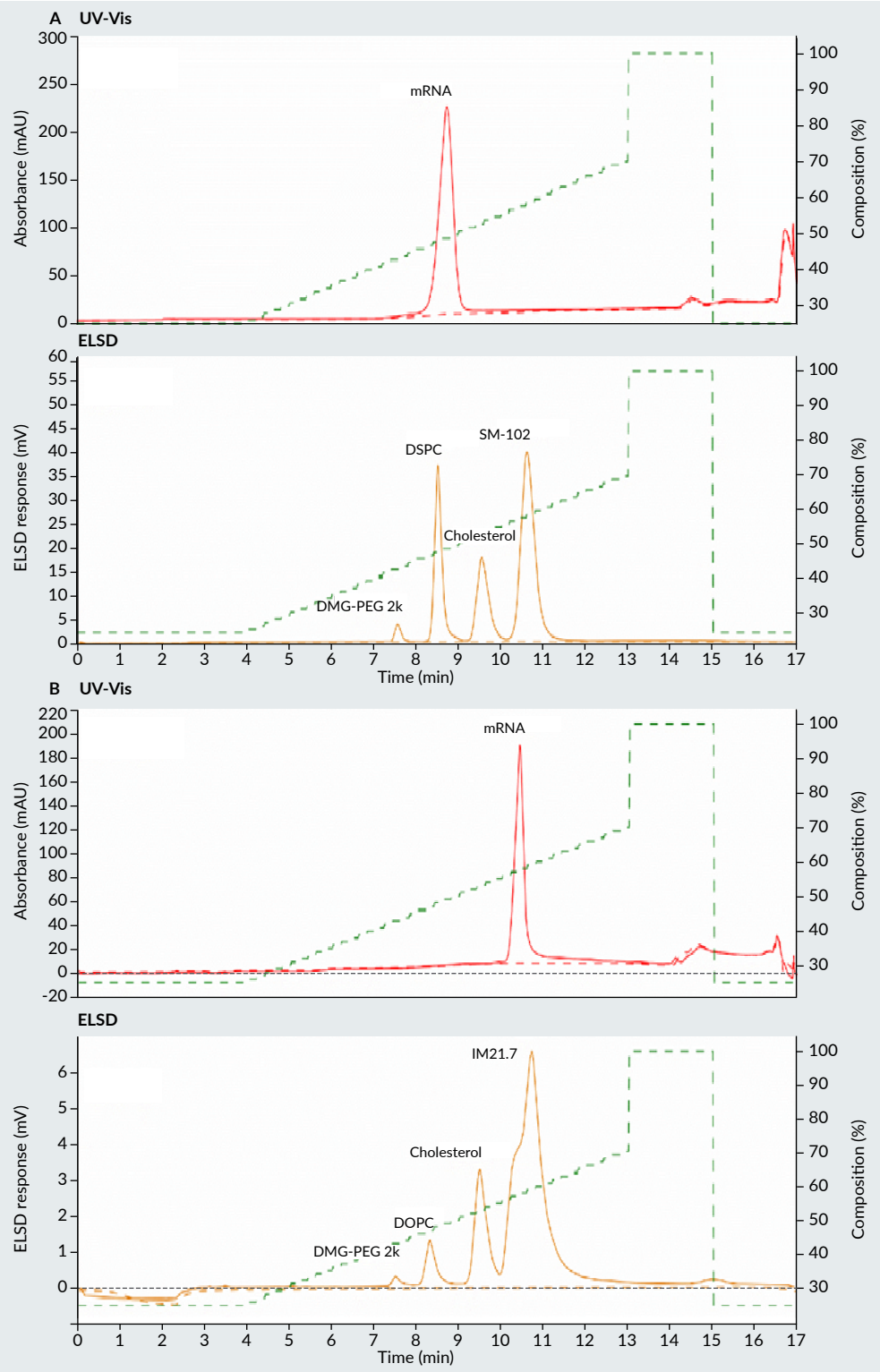
Chromatogram of four lipids used in preparation of LNP with (A) ionizable lipid, and (B) cationic lipid.



Upper part of the chromatogram represents UV-Vis detector signal and lower part ELSD signal. LNP: Lipid nanoparticles; ELSD: Evaporative light scattering detectors.

FIGURE 2

Chromatogram of two LNP samples prepared with (A) ionizable lipid, and (B) cationic lipid.



Upper part of the chromatogram represents UV-Vis detector signal and lower part ELSD signal.

TABLE 1

Validation results.

Sample	Retention time (min)	Calibration curve	R2 mRNA	LOD (µg) mRNA	LOQ (µg) mRNA	RSD (peak areas; %) n = 6	Recoveries (%) n = 6 (ELSD)	Recoveries RSD (%) n = 6 (ELSD)
mRNA	9.1	y=2602× – 56	0.9999	0.01	0.07	N/A	N/A	N/A
IM21.7c	10.4	$y=0.7171x^2+4.7673x-3.1714$	0.9997	0.56	0.75	N/A	N/A	N/A
DOPC	8.2	$y=1.2365x^2+10.886x-8.8627$	0.9989	0.57	0.60	N/A	N/A	N/A
SM-102	10.6	$y=1.423x^2+5.5894x-1.9832$	0.9994	0.44	0.55	1.0	91.0	0.5
DSPC	8.4	$y=0.9497x^2+28.134x-28.049$	0.9998	0.62	0.69	0.8	95.1	0.3
Cholesterol	9.5	$y=2.3862x^2+18.396x-23.192$	0.9991	0.56	0.62	1.7	94.5	0.8
DMG-PEG2k	7.5	$y=1.0009x^2+13.824x-15.694$	0.9991	0.54	0.58	1.2	91.6	0.5

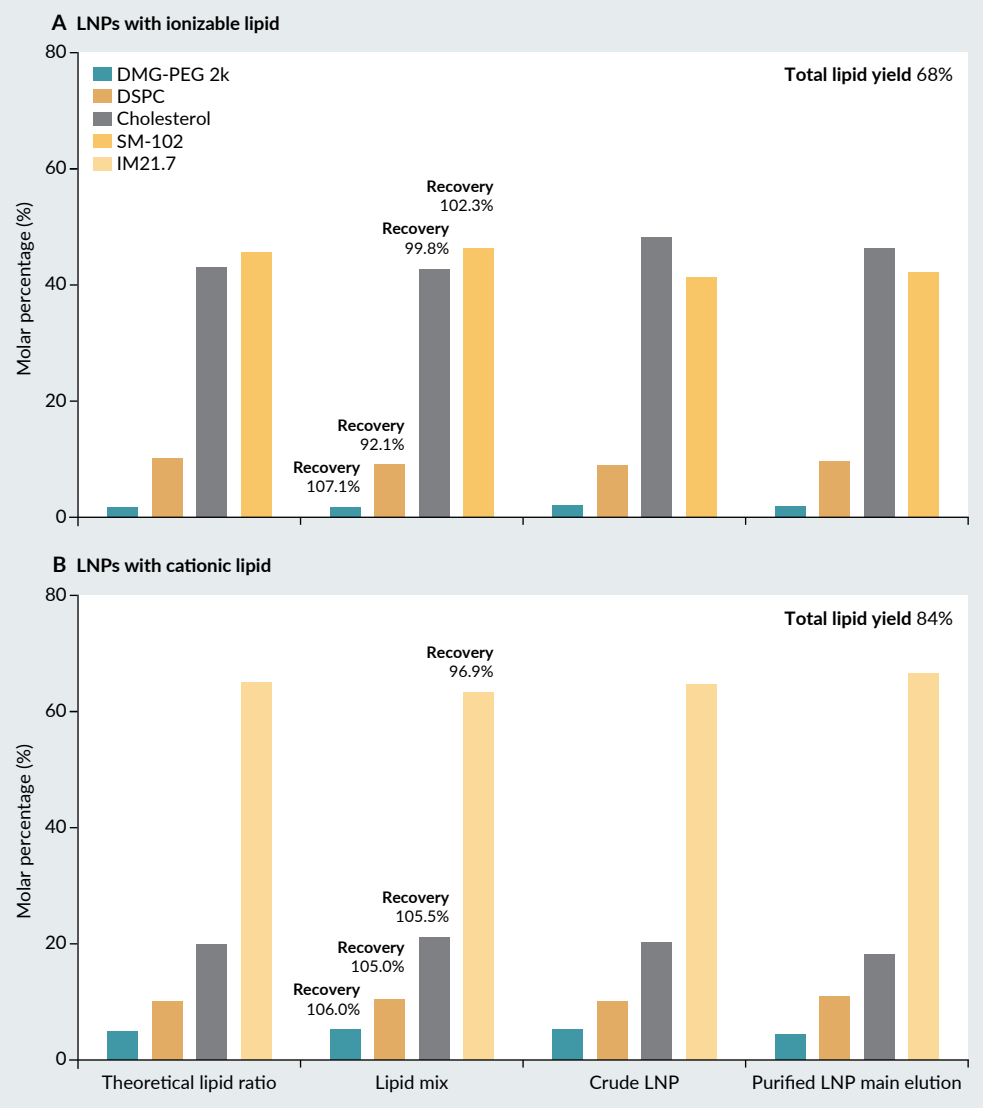
model (ax^2+bx+c) (Table 1). In the case of UV detection of mRNA, normal linear model was used ($y=kx+n$) (Table 1). The precision of the method was evaluated by analyzing standard mixtures of the four lipids composing LNP sample with ionizable lipid the at 1 µg/mL each in ethanol. The relative standard deviation (RSD) values of the peak areas measured by ELSD from six consecutive injections of the same standard lipid mixture were calculated to check the method's repeatability (Table 1). In order to assess the accuracy of the method, mixtures of the four lipids composing LNP sample with ionizable lipid were prepared at known concentrations of 1 mg/mL in ethanol. The recoveries, expressed as a percentage, between the known concentrations and the calculated concentrations of lipids, based on the calibration curves, were determined (Table 1).

Method application for LNP composition assessment

The applicability of the validated chromatographic method for lipid and mRNA quantification was evaluated by analyzing six different LNPs formulations. Three LNPs were prepared with ionizable lipids, and three with cationic lipids with addition of phospholipid, cholesterol and pegylated lipid. In addition to the different lipid compositions, the lipid ratios varied among the formulations. Samples of LNPs were obtained at various stages of preparation, including the lipid mix injected into the microfluidics machine, the crude LNP sample directly from the microfluidics machine, and the purified LNP sample obtained through tangential flow filtration (TFF). The nature and proportion of lipids must be tailored to each application, necessitating the quantitative analysis of each lipid [23]. Subsequently, the chromatographic method was employed as a quality control tool to monitor changes in lipid ratios of nanoparticles throughout the manufacturing process. Prior to subjecting the lipids to the microfluidic machine, the recoveries (percentage of each lipid compared to the theoretical quantity) were calculated for all lipids, with values ranging from 92.1%–107.1% for LNPs with

FIGURE 3

Molar percentage of the LNP building block lipids in LNP with (A) ionizable lipid, and (B) cationic lipid.



ionizable lipid and from 96.7%–106.0%. This demonstrated the reliability of the developed chromatographic method (Figure 3).

In the case of LNPs prepared with ionizable lipid, the molar percentage of ionizable lipid (SM-102) decreased from 46.5%–41.2% during microfluidic mixing. In contrast, the molar percentage of cholesterol increased from 42.9%–48.2%. The molar percentages of phospholipid and pegylated lipid remain unchanged during microfluidic mixing. TFF filtration does not affect the lipid ratio of the LNPs. The manufacturing process of the LNPs resulted in a loss of between 32% of the total lipid concentration (Figure 3), without inducing significant changes in lipid molar ratios. This loss of lipids was probably due to the elimination of lipids not involved in the lipid nanoparticles, which were able to pass through the TFF membrane.

A different observation was made with LNPs prepared with cationic lipid. In this case, the molar percentage of cationic lipid increased slightly from 63.0%–66.5% during microfluidic mixing and TFF filtration. Consequently, a slight decrease in the molar

percentage of cholesterol is observed. Again, the molar percentage of phospholipid and pegylated lipid remained the same. The loss of lipids was not as pronounced as in the case of ionizable lipids, only about 16% of the total lipid concentration was observed (Figure 3).

CONCLUSION

This study presents the development and validation of a novel reverse phase liquid chromatography method for the quantitative analysis of lipids and nucleic acids in LNPs. The method, which utilizes an evaporative light scattering detector and CIMac C4 HLD monolithic column, enables direct injection of LNP formulations, allowing simultaneous separation and quantification of both lipid components and nucleic acid without the need for sample pre-treatment. The utilization of a monolith CIMac C4 HLD column enabled the developed method to achieve effective separation and distinct detection of all lipid constituents. The method was demonstrated to be highly sensitive, linear, precise, and accurate, meeting the specifications included in ICH guidelines, thereby proving its reliability for routine analytical applications. Furthermore, the method revealed its ability to track changes in lipid composition throughout the LNP manufacturing process, thereby ensuring the stability and consistency of LNP formulations.

The application of the method to a range of LNP formulations, including both ionizable and cationic lipids, demonstrated its robustness in detecting differences in lipid ratios and mRNA. This capability highlights the potential of the method as a critical quality control tool, aiding in the reproducibility and efficacy of LNP-based therapeutics. Consequently, this advanced analytical approach supports the ongoing development and optimization of LNPs for diverse therapeutic applications, potentially leading to significantly improved LNP-based therapeutics.

REFERENCES

1. Hou X, Zaks T, Langer R, Dong Y. Lipid nanoparticles for mRNA delivery. *Nat. Rev. Mater*. 2021; 6(12), 1078–1094.
2. Li Y, Ye Z, Yang H, Xu Q. Tailoring combinatorial lipid nanoparticles for intracellular delivery of nucleic acids, proteins, and drugs. *Acta Pharm. Sin. B* 2022; 12(6), 2624–2639.
3. Schlich M, Palomba R, Costabile G, *et al.* Cytosolic delivery of nucleic acids: the case of ionizable lipid nanoparticles. *Bioeng. Transl. Med.* 2021; 6, 2.
4. Li M, Li Y, Li S, *et al.* The nano delivery systems and applications of mRNA. *Eur. J. Med. Chem.* 2022; 227, 113910.
5. Kulkarni JA, Darjuan MM, Mercer JE, *et al.* On the formation and morphology of lipid nanoparticles containing ionizable cationic lipids and siRNA. *ACS Nano* 2018; 12(5), 4787–4795.
6. Hald Albertsen C, Kulkarni JA, Witzigmann D, Lind M, Petersson K, Simonsen JB. The role of lipid components in lipid nanoparticles for vaccines and gene therapy. *Adv. Drug Deliv. Rev.* 2022; 188, 114416.
7. McLaren DG, Miller PL, Lassman ME, Castro-Perez JM, Hubbard BK, Roddy TP. An ultraperformance liquid chromatography method for the normal-phase separation of lipids. *Anal. Biochem.* 2011; 414(2), 266–272.
8. Abreu S, Solgadi A, Chaminade P. Optimization of normal phase chromatographic conditions for lipid analysis and comparison of associated detection techniques. *J. Chromatogr. A* 2017; 1514, 54–71.
9. Hutchins PM, Barkley RM, Murphy RC. Separation of cellular nonpolar neutral lipids by normal-phase chromatography and analysis

by electrospray ionization mass spectrometry. *J. Lipid Res*. 2008; 49(4), 804–813.

10. Peterson BL, Cummings BS. A review of chromatographic methods for the assessment of phospholipids in biological samples. *Biomed. Chromatogr.* 2006; 20(3), 227–243.

11. Lin JT, Mckeon TA, Woodruff CL, Singleton JA. Separation of synthetic phosphatidylcholine molecular species by high-performance liquid chromatography on a C column. *J. Chromatogr. A* 1998; 824(2), 169–174.

12. Cajka T, Fiehn O. Comprehensive analysis of lipids in biological systems by liquid chromatography-mass spectrometry. *Trends Analyt. Chem.* 2014; 61, 192–206.

13. Suchocka Z, Gronostajska D, Suchocki P, Pachecka J. New HPLC method for separation of blood plasma phospholipids. *J. Pharm. Biomed. Anal.* 2003, 32, 859–865.

14. Knittelfelder OL, Weberhofer BP, Eichmann TO, Kohlwein SD, Rechberger GN. A versatile ultra-high performance LC-MS method for lipid profiling. *J. Chromatogr. B* 2014; 1, 119–128.

15. Harkewicz R, Dennis EA. Applications of mass spectrometry to lipids and membranes. *Annu. Rev. Biochem*. 2011; 80(1), 301–325.

16. Cajka T, Fiehn O. Comprehensive analysis of lipids in biological systems by liquid chromatography-mass spectrometry. *Trends Analyt. Chem.* 2014; 61, 192–206.

17. Grit M, Crommelin DJA, Lang J. Determination of phosphatidylcholine, phosphatidylglycerol and their lyso forms from liposome dispersions by high-performance liquid chromatography using high-sensitivity refractive index detection. *J. Chromatogr. A* 1991; 585(2), 239–246.

18. Zhong Z, Ji Q, Zhang JA. Analysis of cationic liposomes by reversed-phase HPLC with evaporative light-scattering detection. *J. Pharm. Biomed. Anal*. 2010; 51(4), 947–951.

19. Bender V, Fuchs L, Süss R. RP-HPLC-CAD method for the rapid analysis of lipids used in lipid nanoparticles derived from dual centrifugation. *Int. J. Pharm. X* 2024; 7, 100255.

20. Kim KH, Lee JE, Lee JC, *et al.* Optimization of HPLC CAD method for simultaneous analysis of different lipids in lipid nanoparticles with analytical QbD. *J. Chromatogr. A*. 2023; 1709, 464375.

21. Yu X, Yu C, Wu X, *et al.* Validation of an HPLC-CAD method for determination of lipid content in LNP-encapsulated COVID-19 mRNA vaccines. *Vaccines (Basel)* 2023; 11(5), 937.

22. EMA. *ICH Q2(R2) Guideline on Validation of Analytical Procedures*. 2024; European Medicines Agency. https://www.ema.europa.eu/en/ich-q2r2-validation-analytical-procedures-scientific-guideline.

23. Eygeris Y, Gupta M, Kim J, Sahay G. Chemistry of lipid nanoparticles for RNA delivery. *Acc. Chem. Res.* 2022; 55(1), 2–12.

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Overcoming challenges in gene therapy analytics

Anthony J Blaszczyk and Ana Carreras González

The range of available AAV serotypes with differing tropisms offers the potential for precisely targeted *in vivo* gene delivery. However, to sustain a healthy rate of growth of the AAV platform, robust, GMP-compliant analytical methods and characterization protocols are a necessity, with a key critical quality attribute being empty/full capsid ratio identification. In this article, based on a webinar symposium, various methods available for AAV vector characterization are explored. These include several methods commonly used in empty/full AAV particle characterization and an assessment of recombinant AAV (rAAV) purity characterization via capillary electrophoresis sodium dodecyl sulfate (CE-SDS).

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THE LANDSCAPE OF ANALYTICAL CHARACTERIZATION OF AAV EMPTY/FULL PARTICLES BY ANTHONY J BLASZCZYK

USP standards for biologics

The *United States Pharmacopeia* (*USP*) is an independent, scientific, non-profit organization that has been providing public standards to support the quality of medicine and improve public health for over 200 years [1]. *USP*'s efforts help ensure public standards are available to help verify the quality and safety of biologics on a global basis, while their documentary standards provide procedures and acceptance criteria to support medicines, including cell and gene therapies. *USP* offers three general types of public standards: monographs, general chapters, and physical reference standards.

- Monographs are specifications for pharmaceutical articles in commerce, from release to product shelf life;
- General chapters can be divided into two different sections: applicable general chapters numbered <1,000, and informational general chapters numbered >1,000. Applicable general chapters are often referred to as compendial methods or chapters and contain validated methods with associated reference standards, where applicable. These are recognized by regulatory authorities such as the US FDA. Informational chapters offer best practices, suggestions, and guidelines for how different practices should be used. These are written based upon input from experts in the field and further vetted through public comments;
- Physical reference standards provide traceable standards to demonstrate the broad-based acceptability of procedures.

In the context of AAV, *USP* has established an AAV Gene Therapy Expert Panel to develop a *USP* general chapter. This panel initiated work in June 2022 and a first draft of the chapter, which will be published in *USP* Pharmacopeial Forum, is expected in 2024. The chapter outline includes vector characterization, materials, manufacturing, formulation and final presentation, control strategy, stability, and comparability.

AAV empty/full particle method comparison

USP has evaluated several methods commonly used in empty/full AAV particle characterization, including size exclusion chromatography with multi-angle light scattering (SEC-MALS), digital PCR (dPCR)/ELISA, UV-spectrophotometry, charge detection mass spectrometry (CD-MS), CE-SDS, cIEF, and mass photometry. Both AAV5 and AAV8 serotypes were tested, each having a transgene of 4.3 kb. To best assess the methods, full particles, empty particles, and a 50:50 mix of full and empty particles were analyzed. An overview of the results for each method is given in Table 1.

SEC-MALS analysis

SEC-MALS is performed using (ultra)-high-performance liquid chromatography ([U]HPLC), and data are captured with UV and MALS detectors. SEC-MALS is a high-throughput technique that can be easily implemented into a GMP environment. Like many other physicochemical empty/full methods, a downside of SEC-MALS is the inability to resolve intermediate mass species, commonly known as partially full.

The instrument used for these tests was a Waters ACQUITY UPLC® with a Wyatt DAWN™ MALS detector. SEC separation was accomplished with a Wyatt Silica SEC Protein Column (4.6×300 mm, 5 µm, 500 Å). The data collected using SEC-MALS showed that the empty sample for both AAV5 and AAV8 contained a very low percentage full, as expected. SEC-MALS calculated AAV5-full to be 80% full and AAV8-full to be 89.3% full, and the mixed samples to be 40.5% and 45.4% for AAV5 and AAV8, respectively.

dPCR/ELISA analysis

Both dPCR and ELISA are commonly used methods, often utilized in the GMP environment. Genome titer PCR results, which determined the amount of transgene in AAV, were coupled with capsid titer results, which determined the capsid concentration. Using dPCR/ELISA to calculate empty/full is easily implemented, because it utilizes existing data from two assays that are already routinely performed in GMP environments as part of release testing. However, as with SEC-MALS, they do not resolve intermediate mass species. Furthermore, this method is known to be more prone to variability

TABLE 1

Summary of empty/full AAV5 and AAV8 particle data using various characterization methods.

%Full AAV	dPCR/ELISA	SEC-MALS	CD-MS	Simple Western	cIEF	UV (A260/280)	Mass photometry
AAV5-empty	0.0	0.3	0.3	0.8	6.3	9.1	3.3
AAV5-full	91.0	80.0	96.3	90.7	88.2	87.5	95.7
AAV5-mix	56.0	40.5	62.9	55.8	53.2	46.7	60.5
AAV8-empty	0.0	0.0	0.7	8.3	9.1	9.5	1.3
AAV8-full	89.0	89.3	98.1	97.8	93.7	96.7	97.1
AAV8-mix	45.0	45.4	47.7	58.6	40.4	52.1	54.7

CD-MS: Charge detection mass spectrometry; dPCR: Digital PCR; SEC-MALS: Multi-angle light scattering.

because the variability from two independent methods will be incorporated into the final results.

Data collected using this method showed a low percentage of full for the AAV5-empty and AAV8-empty samples. AAV5-full and AAV8-full were determined to be 91% and 89% full, respectively, while AAV5-mix and AAV8-mix were 56% and 45% full, respectively.

UV spectrophotometry (260/280) analysis

Spectrophotometry using 260/280 nm ratio is a high-throughput and easily implemented technology in a GMP environment, as most GMP labs perform UV-vis analysis for concentration determinations. Using an analysis that was first published by Sommer *et al.* in 2003, the percent of full AAV can be calculated using the 260 nm/280 nm ratio. Although this method is rapid and has low volume requirements, it is most often used as a characterization assay, as it is sensitive to buffer/sample interference that could greatly impact the data.

The SoloVPE System was used to measure the absorbance at 260 nm and 280 nm. Data collected showed a slightly higher percentage full in the AAV5 and 8 empty samples compared with other methods discussed, although these were still low overall. AAV5-full and AAV-8-full were 87.5% and 96.7% full, respectively, and AAV5-mix and AAV8-mix were 46.7% and 52.1% full, respectively.

CD-MS analysis

CD-MS allows mass/charge analysis of a single molecule, which allows relative assessment of capsid content of AAV particles. This method reports the m/z of all the different AAV species in a sample, which will increase with additional transgene packaged inside of the capsid. This method is very sensitive and does allow resolution of intermediate species.

The results for this method showed a high percentage full for AAV5-full and AAV8-full of 96.3% and 98.1%, respectively. AAV5-mix and AAV8-mix were 62.9% and 47% full, respectively. The empty samples for both serotypes were both very low, having <1% full capsid.

Simple Western analysis

Simple Western analysis (Simple Western) was performed by the automated ProteinSimple's Peggy Sue™ instrument. No sample pre-treatment is required, and Peggy Sue can analyze complex samples, is quantitative over a 3–4-log dynamic range, and has very low material requirements. Using this method, the empty/full ratio can be analyzed using antibodies specific to the transgene and the capsid to generate a standard curve and determine the percentage full for any unknowns using a linear line.

Data collected from this method compares well to the other methods tested, with AAV5-full and AAV8-full at 90.7% and 97.7% full, respectively, and AAV5-mix and AAV8-mix at 55.8% and 58.6% full, respectively.

icIEF and CE-SDS analysis

For icIEF and CE-SDS analysis, another ProteinSimple instrument, Maurice™ (www.bio-techne.com/instruments/ice), was used. This instrument allows for direct detection and rapid data in as little as 12 minutes per sample. No sample pretreatment is required, and the method is relatively easy to implement in a GMP environment. The method is quantitative over a 1–2-log dynamic range. The Maurice E/F assay leverages dual-channel detection using absorbance, where both DNA and protein contribute to the signal, and native fluorescence, where only protein directly contributes. Standard curves are generated, from which the amount of DNA and protein are calculated, allowing for further calculation of percent full or empty.

Data collected were comparable to previous methods, with AAV5-full and AAV8-full at 88.2% and 93.7% full, respectively.

Mass photometry

Mass photometry uses light scattering principles to determine the mass of single molecules in solution. Using a Referyn Samux^{MP}, the mass of AAV samples can be determined, which can be used to calculate the abundance of empty, partial, and full capsids. The Refeyn Samux^{MP} quick protocol was used as a generic method. Mass photometry has many advantages, including low sample volume requirements, a large dynamic range, high-throughput performance, and ability to resolve intermediate mass species.

Data collected from this method were comparable to other methods, with AAV5-full and AAV8-full at 95.7% and 97.1% full, respectively, while the empty samples both were calculated to have <3.5% full capsid.

Conclusions

Generally, all methods compare reasonably well from the data generated and discussed in this presentation. However, the precision of these methods cannot be assessed from this study, as all tests were performed in a single laboratory and contained only a single data point (N=1). To better understand the various empty/full methods, a more intricate study needs to be performed that involves multiple laboratories.

In summary, the complexity and diversity of cell and gene therapies presents challenges in the standardization of methods and assays. There is a wide array of commonly used methods for empty/full AAV particle characterization, including SEC-MALS, dPCR/ELISA, UV-spectrophotometry, CD-MS, CE-SDS, cIEF, and mass photometry. *USP* is committed to working with stakeholders to help streamline and expedite the development of safe and effective therapies for patients, including AAV therapies. Opportunities for collaboration with *USP* are always available, whether volunteering for an expert panel or donating methods or other support.

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BREAKING THROUGH THE CHALLENGES OF RAAV PURITY CHARACTERIZATION BY CE-SDS BY ANA CARRERAS GONZÁLEZ

We believe Viralgen's scalable rAAV production platform represents a powerful tool for GMP grade gene therapy drug manufacturing, especially using the serotypes rAAV6, rAAV8, and rAAV9. The platform has already manufactured over 1000 batches at 2 L, 50 L, 250 L, 500 L and 2000 L scale and is

providing services to more than 50 customers globally. Viralgen's technology is based on the Pro10™ cell line, which is a unique, high-yield HEK293-derived cell line specially engineered for rAAV production. This cell line is serum-free, grows in cell suspension, can be easily scaled up, and offers high transfection capacity.

Purity assessment of rAAV by CE-SDS

The purity assessment of rAAV vectors involves VP1, VP2, and VP3 capsid protein determination and aims to identify any process and host-cell-related impurities. The Maurice platform provided by Bio-Techne® can assess the purity of rAAV by capillary electrophoresis under reduced conditions (CE-SDS), separating proteins by size with capillary selective focusing.

Historically, rAAV vector purity has been widely assessed by SDS-PAGE silver staining, in which VP1, VP2, and VP3 capsid proteins are present as gel bands, with VP3 being the most abundant. However, this analytical method has several shortcomings as it is non-quantitative, has a relatively high degree of user variability, and is a time and reactive-consuming assay. In contrast, CE-SDS is a semi-quantitative technique that presents high reproducibility and offers VP protein ratio determination aside from purity percentage assessment.

When the CE-SDS technique was first developed in Viralgen facilities, the linearity of the method was assessed. During these first method development activities it was found that sample linearity was lost at the highest points of the curve, and therefore, the coefficient of determination did not meet the required standards of accuracy. A subsequent short study discovered that the salt present in the sample buffer was interfering with the electrokinetic injection performed by the instrument.

The Maurice system comprises a cartridge in which the proteins are injected by electrokinetic force. In brief, a voltage is applied into the sample and the negatively charged proteins are introduced in the capillary. Salts present in the sample impair the entry of the proteins and thus cause the loss of linearity in the high points of the standard curve. As a solution, a salt removal step by centrifugal filter units was implemented, and the linearity of the method was recovered, reaching a coefficient of determination over 0.99. As a recommendation, sample salt content must be below 50 mM to achieve a good linearity value for this analytical method.

Based on this result, the CE-SDS method validation for rAAV purity assessment was performed for rAAV8 and rAAV9 serotypes following the ICH-Guidelines Q2(R1) [2]. The outcome is shown in Table 2. The left column of the table shows the qualification parameters assessed, and the obtained results are gathered on the right column. The method achieved a low limit of quantification based on the signal-to-noise response and a good linearity between 2.5×10^{12} vp/mL and 6.13×10^{13} vp/mL. The accuracy values throughout the linearity curve were also within the expected range. The precision determination (repeatability and intermediate precision parameters) obtained low coefficients of variation (CV) for sample replicates performed in different days and by different analysts assessing both peak area and peak % area. Moreover, purity assessment variability was evaluated across six independent runs, giving a CV of <1%, and thus confirming the suitability of the method for the intended use. Overall, rAAV purity assessment performed by CE-SDS meets the criteria set by the FDA and EMA for GMP-compliant analytical method validation.

After accomplishing the validation, another challenge arose: when the method developed for rAAV8 and rAAV9 was applied to other serotypes, such as rAAV2, rAAV3, and rAAV6, almost 70% of the sample was lost (Figure 1). Upon further study, it was discovered that the sample loss happened when centrifuging or removing the salt by

TABLE 2

Purity assessment: CE-SDS method validation results.

CE-SDS validation for rAAV purity assessment (rAAV8 and rAAV9)	
Qualification parameter	**Obtained results**
Specificity	Specific for rAAV
LOQ	S/N 7.17
Linearity	R^2 >0.999
Accuracy	86%–110%
Range	2.5 x 10^{12} VP/mL–6 x 10^{13} VP/mL
Repeatability	%CV <1% area %CV <4 area
Intermediate precision	%CV <1% area %CV <9 area
Purity assessment variability	%CV <1% area

CE-SDS: Capillary electrophoresis sodium dodecyl sulfate.

the centrifugal system units. One trait shared by these three serotypes is that they present a heparin-binding domain on the surface [3]. The main hypothesis to explain the sample loss is that this primary receptor may be interacting with the centrifugal unit´s filter, but this is still being confirmed.

Based on this finding, it was highlighted that the centrifugal filter units might not provide an optimal solution for all rAAV serotypes. In collaboration with Bio-Techne applications science team, a salt removal method based on acetone precipitation was implemented. In short, the sample is mixed with four-times the sample volume of ice-cold acetone, incubated for 1 hour at −20 ºC, followed by a centrifugation step. The supernatant is removed, and the pellet is air-dried before re-suspension in a buffer suitable for the CE-SDS method.

Following this method, the peak signal obtained increased six-fold, and good absorbance and precision values were obtained, as shown in Figure 2. The intermediate precision results for six replicates across three independent runs showed a CV of <3% for peak area and <1% for peak % area. Additionally, the linearity of this method was assessed, achieving a good result within 4.00×10^{12} vp/mL–3.50×10^{13} VP/mL range. Based on these results, we believe we should validate this new CE-SDS based analytical method for rAAV2, rAAV3, and rAAV6 serotypes in the near future.

In conclusion, we believe the CE-SDS analytical method performed by the Maurice instrument is suitable for GMP-compliant rAAV purity assessment, showing excellent linearity and precision parameters, broad sample concentration range, and good accuracy values.

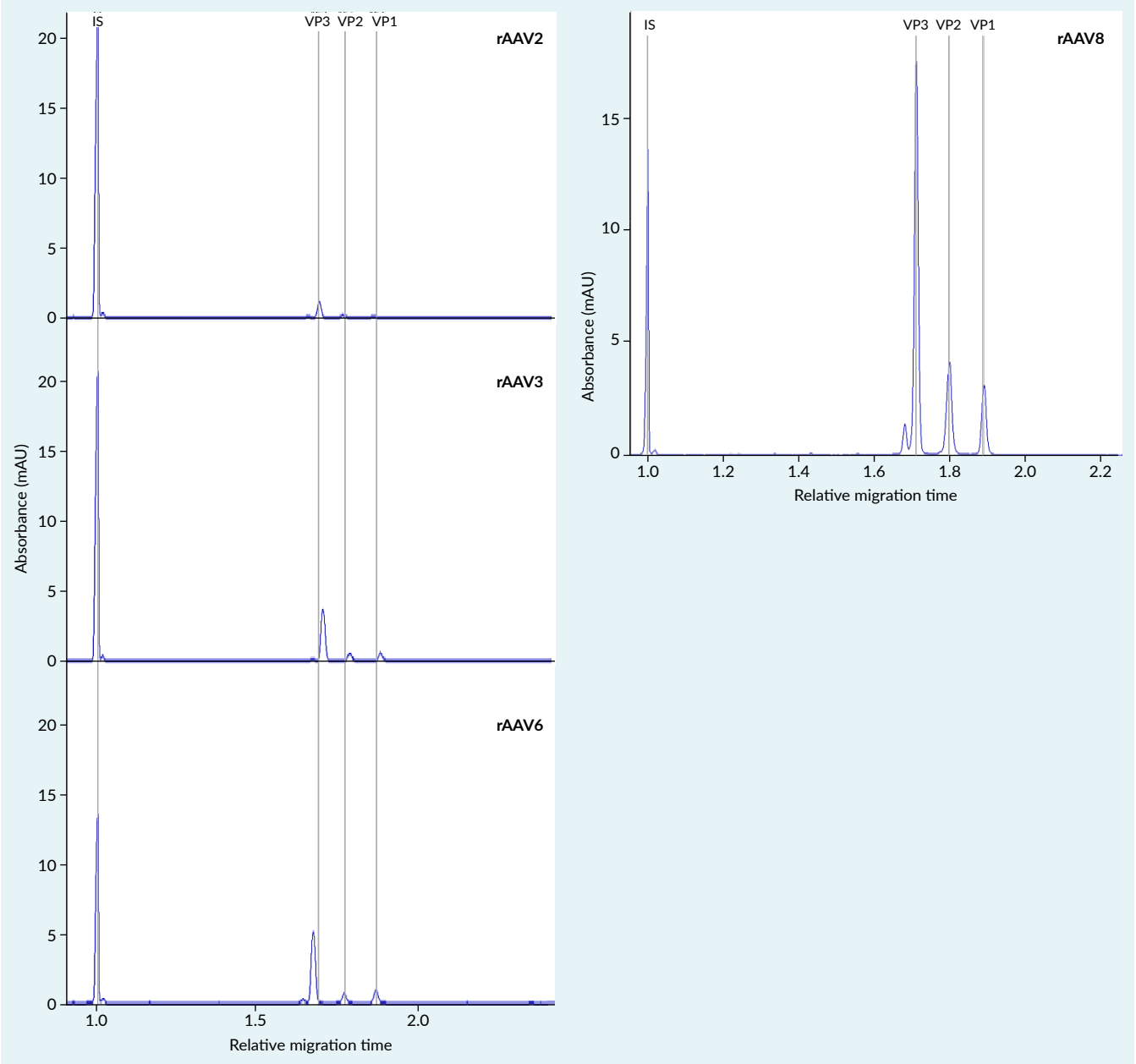
Purity assessment: impurity nature study

After the previous study, the question whether the CE-SDS platform is suitable to study the nature of impurities remained unanswered. By CE-SDS, these impurities appear in the shape of additional peaks aside from the capsid protein peaks, VP1, VP2, and VP3.

The first factor to determine was if DNA is visible when measuring by CE-SDS. The Maurice system was initially developed to monitor the proteins present in a sample by absorbance at 220 nm. This wavelength corresponds to the absorbance of the peptide bond, and it was unknown if DNA was visible at this wavelength. To answer this question, several quantities of plasmid DNA were analyzed by CE-SDS in the Maurice system. As a result, it was seen that not only were there visible peaks, but the

FIGURE 1

Centrifugal system units cause sample losses in rAAV2, rAAV3, and rAAV6 serotypes (left), compared to the rAAV8 serotype (right).



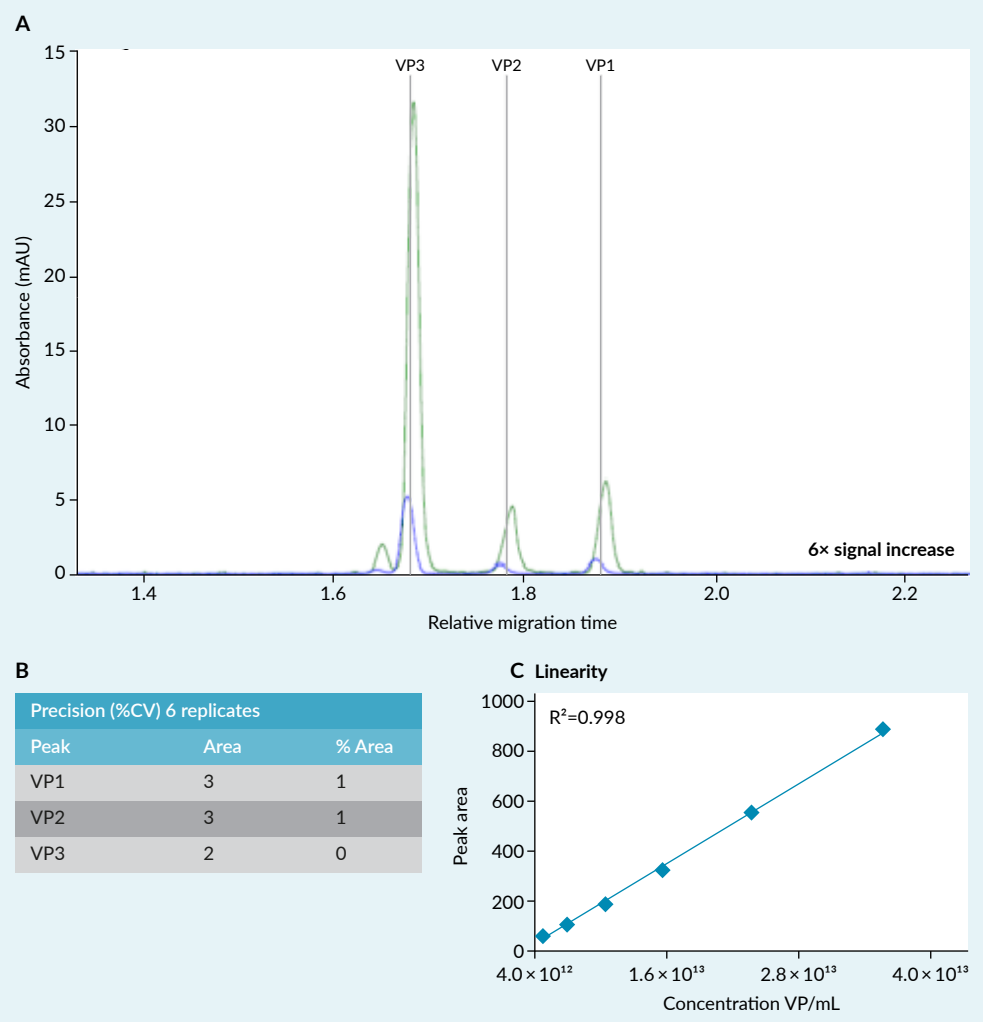
The same sample concentration was loaded in all cases.

DNA showed a linear response based on peak area (Figure 3), demonstrating that the Maurice CE-SDS based analytical method can be used to monitor DNA in addition to proteins.

The second factor to explore was whether the acetone precipitation method for salt removal is suitable for the identification of DNA-related impurities that may be present in a sample, as this technique is widely used to precipitate proteins. To investigate this, a rAAV sample was spiked with plasmid DNA, and both salt removal methods (centrifugal units and acetone precipitation), were compared. The results showed that not only was the DNA precipitated by acetone, but the

FIGURE 2

An alternative method for salt removal for multiple rAAV serotypes.



Precision (%CV) 6 replicates		
Peak	Area	% Area
VP1	3	1
VP2	3	1
VP3	2	0

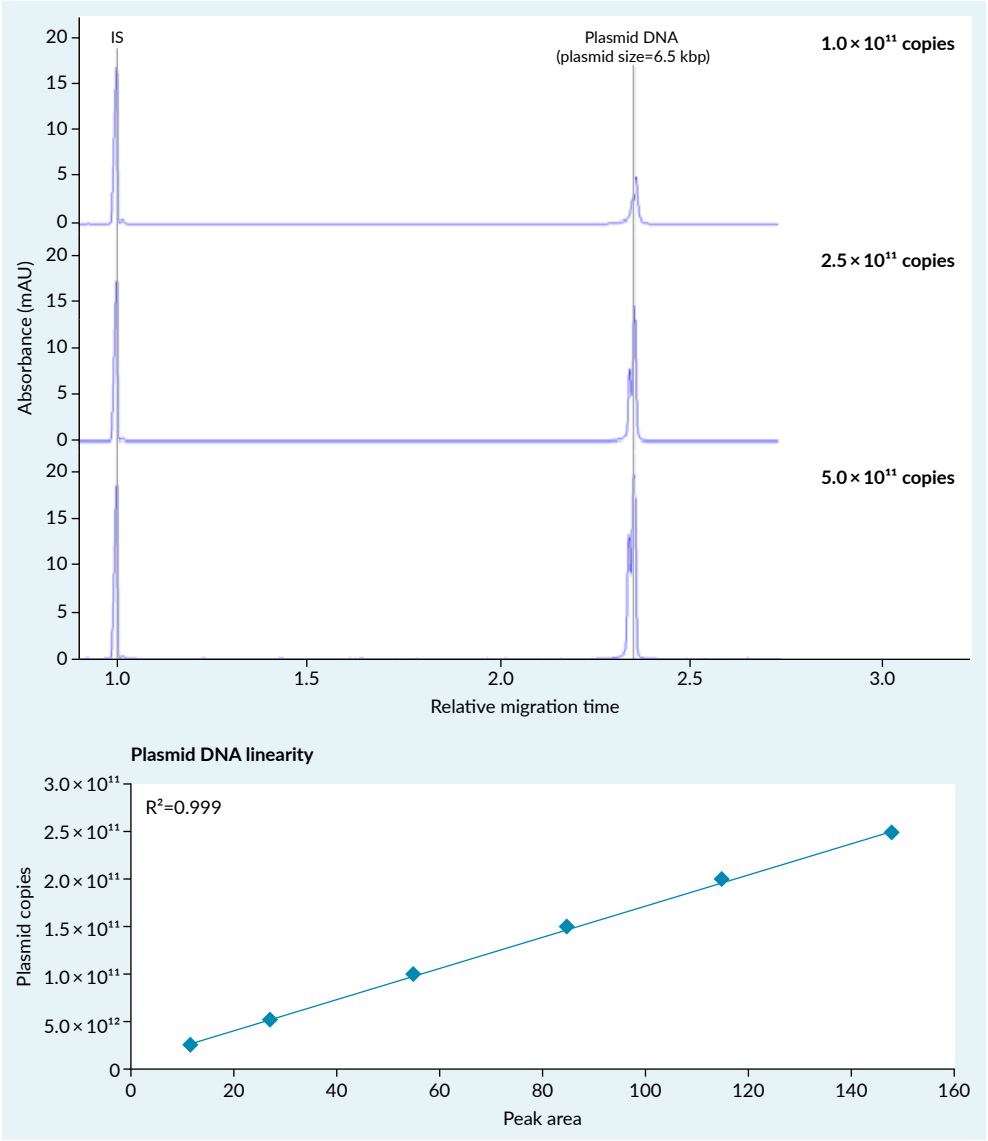
(A) Peak response comparison between the centrifugal system method (blue) and acetone precipitation method (green). **(B)** The precision obtained by the acetone method. **(C)** Linearity assessment of the acetone method.

percentages of the sample belonging to protein and DNA were consistent between both methods. This assay confirmed that acetone precipitation is a suitable salt removal method for purity assessment.

Based on these results, a three-step assay was implemented to determine the nature of the impurity peaks, i.e., whether they belong to protein, free DNA, or encapsidated DNA (Figure 4). The first step of the method consists of a protease treatment followed by a DNA purification column (such as the QIAamp® DNA Mini Kit from Qiagen). If after this treatment the impurity peaks disappear, they are proteins, but if the peaks remain, they are DNA. In this step the peaks belonging to the capsid proteins, VP1, VP2, and VP3, will disappear, confirming the activity of the protease. The second step is a DNase treatment (benzonase); if the impurity peaks disappear, they represent free DNA, but if the peaks remain, they belong to encapsidated DNA. To further confirm the nature of the encapsidated DNA, a heat step treatment to break the capsid was developed, followed by the benzonase treatment. If the peaks disappear, it will confirm the nature of the encapsidated

FIGURE 3

DNA is visible by capillary electrophoresis sodium dodecyl sulfate (right) and shows a linear response based on peak area (left).



DNA. The temperature to break the capsid was optimized based on the capsid melting temperature of each rAAV serotype [4].

Once this strategy was defined, the aim was to first establish the DNase and protease methods. The DNase chosen for this method was benzonase, as it is visible by CE-SDS as a single peak of 32 kDa, as opposed to the DNase I, which presented several peaks. To develop the protocol, plasmid DNA was added to a rAAV sample on a 'free DNA' basis. When this spiked sample was treated with the protease (Proteinase K) followed by the DNA purification column, the peaks belonging to the capsid proteins (VP1, VP2, and VP3) were positively degraded by the protease, and the DNA spike was present (Figure 5). When the same sample was treated with benzonase, the peaks belonging to the capsid proteins were not altered, and the peak belonging to the DNA spike was correctly degraded, confirming the establishment of the first two steps of the method. It is interesting to mention that

FIGURE 4

A three-step method to determine the nature of the impurities.

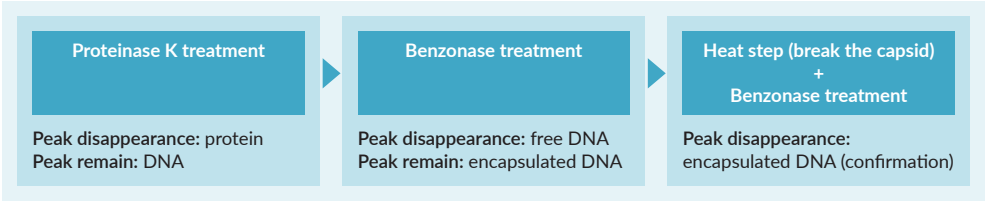
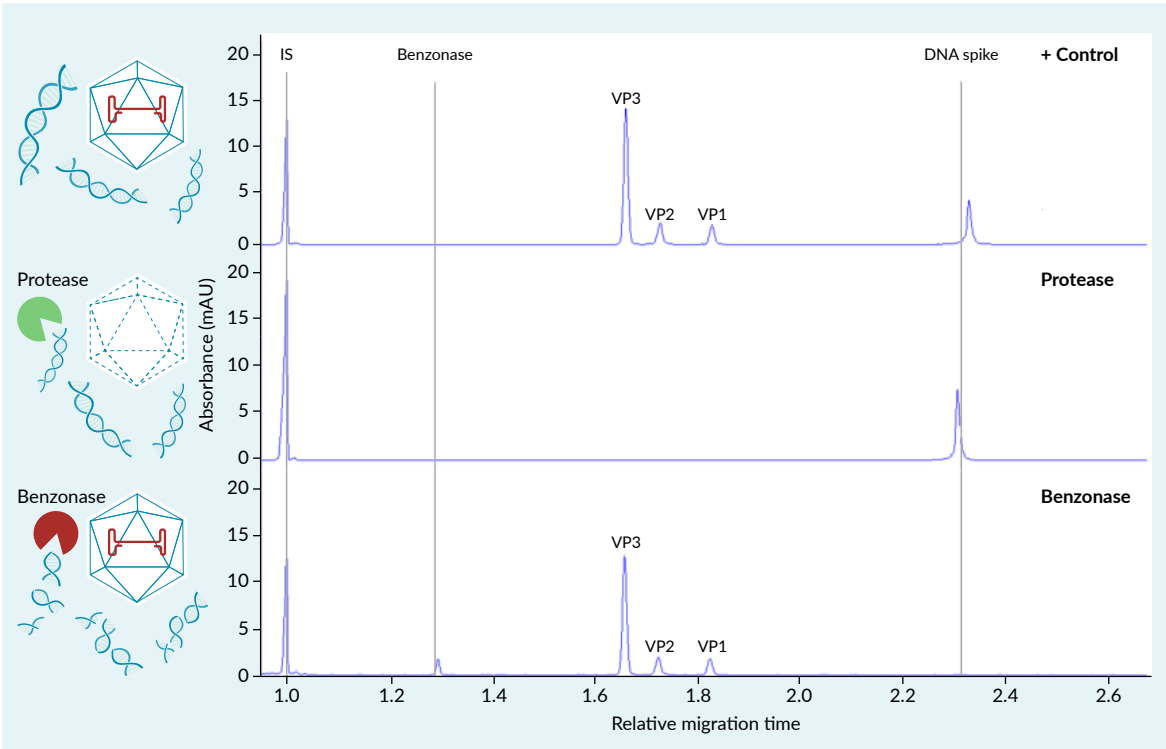


FIGURE 5

Results obtained from a rAAV sample spiked with plasmid DNA, treated by the protease and benzonase steps.



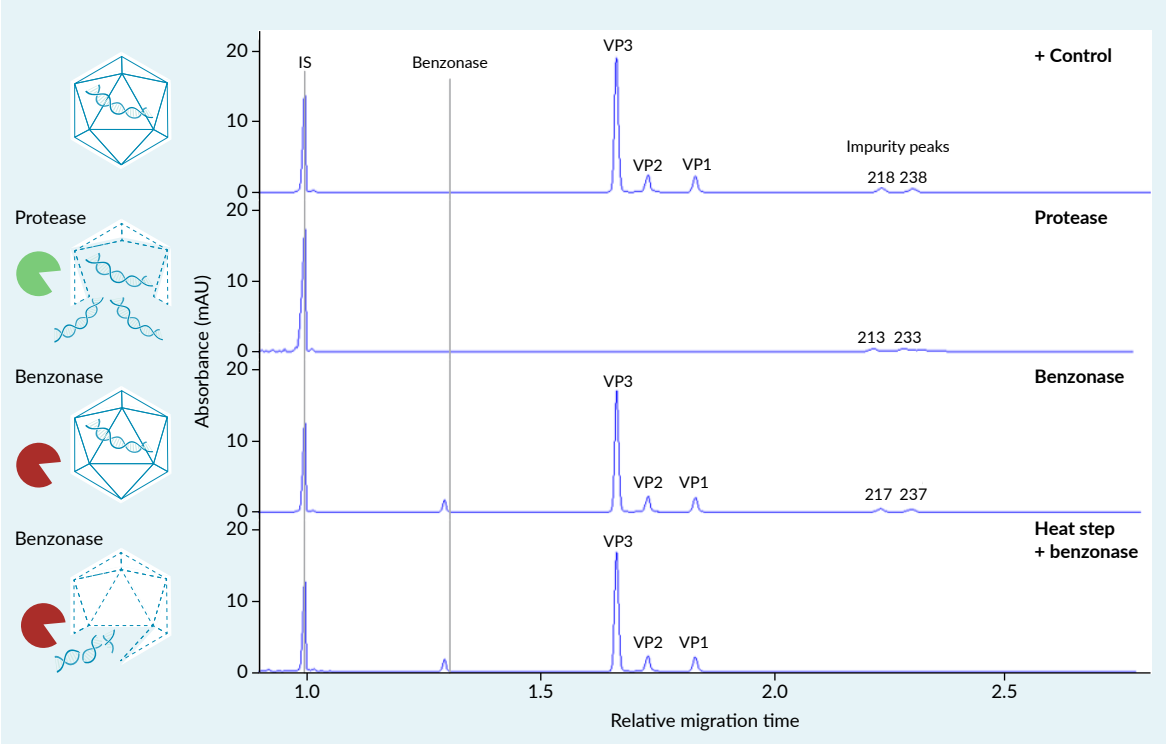
the therapeutic gene harbored in the rAAV, which consist of a single-stranded DNA of 4.5 kb, is not visible by the routine 35 min run performed by the Maurice instrument, as the ssDNA is known to migrate slower than dsDNA by CE-SDS and does not enter the capillary [5].

Having developed the first two steps of the method, the following phase was to establish the heat step to break the capsid. Based on the melting temperature of the capsid [4], the heat step temperature and time were optimized to avoid altering or degrading the capsid proteins. An internal sample with impurity peaks already characterized as encapsidated DNA was used to develop this method.

As shown in Figure 6, when the sample was treated with the protease, the peaks belonging to the capsid proteins disappeared, but those belonging to the encapsidated DNA remained. When the sample was treated with the benzonase, neither the peaks belonging to the capsid proteins nor the encapsidated DNA were altered, suggesting that the DNA is not accessible to the benzonase and thus

FIGURE 6

Results obtained from a rAAV sample showing encapsidated DNA following the three-step method for impurity study.



cannot be degraded. As a confirmation, the heat-step was conducted on the sample to break the capsid, followed by the benzonase treatment. Peaks belonging to encapsidated DNA were positively degraded, confirming its nature. The heat treatment does not affect the integrity of the VP proteins as it only breaks the capsid enough for the DNA to be accessible to the benzonase. We believe these results confirm the development of a method for impurity study of rAAV samples based on the CE-SDS technique.

In summary, we believe these results confirm the development of a method to study the nature of impurity peaks present in recombinant AAV by CE-SDS, capable of distinguishing if they belong to protein, encapsidated DNA, or free DNA. We believe the CE-SDS technique not only successfully assesses sample purity of GMP grade rAAV, but allows a deep study of the impurities present in the sample.

Translation insight

The AAV-driven gene therapy field's recent rapid progress into the commercial sphere has driven innovation from regulatory agencies, with bespoke CMC guidance for the field still emerging, and technology providers alike. In the realm of AAV vector characterization, both novel and repurposed analytical tools will be needed to achieve the improvements in sensitivity, accuracy, and speed required by a sector striving to ensure the consistency and safety of gene therapies.

The development of specific standards will be crucial to the continued maturation and industrialization of AAV manufacturing and QC, particularly as the gene therapy field continues to migrate into increasingly large indications by patient population. Success in this regard will depend in large part on continued effective collaboration between public and private sector stakeholders.

REFERENCES

1. *USP*. Legal Recognition—Standards Categories. *United States Pharmacopeia*. https://www.usp.org/about/legal-recognition/standard-categories.

2. ICH. *ICH-Guidelines Q2(R1), Validation of Analytical Procedures: Text and Methodology*. International Council for Harmonisation. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf.

3. Li C, Samulski RJ. Engineering adeno-associated virus vectors for gene therapy. *Nat. Rev. Genet.* 2020; 21(4), 255–272.

4. Bennett A, Patel S, Mietzsch M, *et al*. Thermal stability as a determinant of AAV serotype identity. *Mol. Ther. Methods Clin. Dev.* 2017; 6, 171–182.

5. Stellwagen E, Stellwagen NC. Electrophoretic mobility of DNA in solutions of high ionic strength. *Biophys. J.* 2020; 118(11), 2783–2789.

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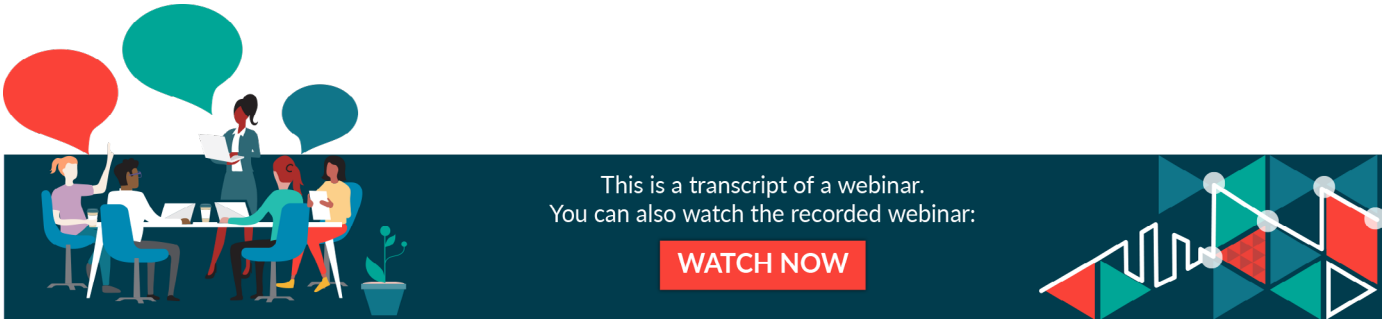

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Global perspectives for cellular and gene therapy development and regulatory expectations: a conference report from the 2023 NIFDS-PMDA-USP Workshop for Advanced Therapies

Ben Clarke, Minkyung Kim, Yoji Sato, Mehrshid Alai-Safar, Masao Sasai, Jongman Yoo, Yoshiteru Kamiyama, Christina G Chase, and Fouad Atouf

The 2023 NIFDS-PMDA-USP Workshop for Advanced Therapies, held in Seoul, South Korea, brought together global regulators, industry representatives, and *United States Pharmacopeia* to discuss the development and regulatory expectations of cellular and gene therapies. These therapies are transforming regenerative medicine and the treatment of cancer and hereditary disorders, necessitating adaptable and flexible regulatory frameworks. The workshop emphasized the importance of inter-agency consensus to accelerate market access for advanced therapies, highlighting challenges and solutions in product quality management, regulatory science, and drug approval processes. To foster scientific advancements and guide the creation of unified regulatory standards, organizers selected topics such as CAR-T therapy, advanced 3D bioprinting, and iPSC-derived cardiomyocyte patches. These sessions focused on quality control strategies, manufacturing comparability, and pre-clinical safety studies. The event underscored the need for ongoing dialogue and collaboration to ensure the safe and effective delivery of these innovative treatments globally.

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Advanced Therapies are an emerging class of medicines that are revolutionizing regenerative medicine and the treatment of cancer and hereditary disorders. Ensuring the global availability of these new therapies requires adaptation and flexibility of existing national regulatory frameworks and consensus-finding between regulatory agencies. Finding consensus among regulatory agencies is important for accelerating market access to advanced therapies. For product developers and manufacturers seeking approval in multiple countries, the unique requirements of individual agencies pose a challenge.

An international group of regulators, industry representatives, and standards development organization convened in Seoul, South Korea with the objective of finding commonality across regulatory agencies. On November 30 and December 1, 2023, the NIFDS-PMDA-USP Workshop for Advanced Therapies was co-organized by the National Institute of Food and Drug Safety Evaluation (NIFDS) of South Korea, the Pharmaceuticals and Medical Devices Agency (PMDA) of Japan, and the *US Pharmacopeia (USP)*. Topics were selected by the organizers to discuss scientific advancements and to inform their efforts to develop common regulatory expectations in this area. The topics include considerations for CAR-T therapies and the recent innovations in organoids, 3D tissue culture, and 3D printing. The invited speakers provided global perspectives on the manufacture of cell, gene, and tissue-engineering products and highlighted key issues in regulatory science and drug approval in different countries.

A conference report detailing sessions 1 and 2 of the workshop was published on June 4, 2024 [1]. This commentary details sessions 3 and 4 of the workshop and provides expert perspectives on the conference.

Opening remarks from leaders of the three sponsoring organizations affirmed the importance of advanced therapies to patients with unmet medical needs and highlighted a mutual objective of increasing their availability. Executive Director Hiroyuki Arai of the Japanese PMDA noted that advanced therapy innovation is taking place worldwide and emphasized the contributions of Japanese, Korean, American, and European product developers and institutions. Many therapies are developed first for a single domestic market and later begin the market-approval process for other regulatory agencies. The mechanisms for seeking near simultaneous approval through a joint market application can be improved through continuing interagency consensus building thus making these therapies widely accessible early on.

The opening remarks of Director General Younjoo Park of South Korea's NIFDS highlighted the intensity of global research and development efforts for advanced therapies and supporting cutting-edge technologies. To evaluate these new technologies and approaches, agency reviewers are quickly acquiring proficiency through additional training, and agencies are recruiting staff with relevant expertise. With the growing number and diversity of therapies in clinical trials, agency capability building at NIFDS and internationally will continue to be a high priority.

The representative of the *USP*, Senior Scientist Ben Clarke, echoed Dr Arai's assessment of the outstanding clinical efficacy that has been demonstrated by cellular and gene therapies. By addressing the longstanding unmet medical needs of patients with inherited and rare diseases, advanced therapies are transforming lives and giving hope to patients. For this emerging class of medicines, it will be critical to set strong quality expectations that are aligned across the world. Finding consensus in

control strategies and developing internationally available reference standards are two ways to support the quality of advanced therapies.

Strong product quality management is one of the key aspects of a product that regulators must evaluate. Dr Arai noted that advanced therapies have some unique and less well-understood scientific and clinical characteristics that could present a safety concern. Another goal of the workshop was to strengthen the understanding of advanced therapies by providing a common platform for regulators, academia, and industry to share their experience and knowledge. Information sharing is critical for identifying emerging issues, finding solutions to shared challenges, and building robust global collaborations that will ultimately bring transformative medicine to critically ill patients.

Director Park also addressed the gap between developed and developing countries in advanced therapy market access, emphasizing that closing this gap will increase access to new therapies. All stakeholders should be strongly encouraged to pursue the shared goals of accelerating innovation and increasing market access. Director Park also emphasized that innovation should not compromise patient safety and public trust in medicine.

SESSION ON EARLY DEVELOPMENT AND QUALITY CONTROL STRATEGIES

Expert speakers shared their experiences and knowledge of cell therapy development and the challenges of quality control in a session moderated by Dr Shinichi Noda (PMDA).

CAR-T therapy

The first speaker, Dr Mehrshid Alai-Safar (VP, Global Regulatory CMC, Kite, a Gilead Company), focused on product quality for CAR-T autologous gene-modified cell therapy. The manufacturing process for a CAR-T starts with leukapheresis to collect an individual patient's blood cells, shipment of the cells to a manufacturing site for processing (purification, gene modification, expansion, and testing), and shipment back to the clinical site for administration to the patient. Since a CAR-T product is a patient-specific therapy for critically ill patients may not survive a delay in treatment due to manufacturing failure or quality issues. Kite has met these challenges by internally managing all aspects of the manufacturing and quality system that spans multiple clinical and manufacturing sites across multiple countries. Ensuring the tight control of the quality of every batch has allowed Kite to scale an autologous therapy to over 16,000 patients.

One resource, the ICH Q8(R2) guideline, was highlighted as being particularly suited to meeting the challenges of CAR-T product development. ICH Q8 describes a product development process that integrates quality principles. It begins with the establishment of a Quality Target Product Profile (QTPP), then recommends understanding product Critical Quality Attributes (CQAs), and then the functional relationship between CQAs and the manufacturing process Critical Process Parameters (CPPs). For cell therapies, potency CQAs are often the most difficult to understand due to their complex mechanisms of action and the lack of models to evaluate long-term persistence and functionality within the patient. Equally difficult is the linking of CQAs to the CPPs that influence them due to the variability of patient-derived starting materials and the confounding of starting material attributes with patient characteristics. Once CQAs have been identified, the specifications for each CQA are established by increasing product understanding through extensive product characterization, the implementation of multiple orthogonal potency assays for functionally overlapping CQAs, and statistical analysis of the clinical effectiveness of each batch. An ICH Q8-informed product development process helped Kite to build a robust manufacturing process and a strong quality system.

Dr Alai-Safar concluded with a manufacturing comparability case study. It is highly likely that product developers will need to

make manufacturing process changes during clinical trials and will need to justify the comparability of the pre-change and post-change products. It may also be necessary to establish comparability across multiple manufacturing changes. Comparability studies are most manageable with a pre-defined and well-powered statistical approach (e.g., equivalence) that is enabled by retaining a sufficient number of samples across the entire product lifecycle.

Advanced 3D bioprinting product combined with stem cells

Professor Sung Won Kim (The Catholic University of Korea College of Medicine) shared his experience developing 3D cellular and tissue bioprinting products for human clinical trials. Advancements in bioprinting can allow the incorporation of organic substances, synthetic polymers, and metal, as well as living cells, into patient-specific products. These innovative and complex products can be used to regenerate, repair, or replace human tissue.

Professor Kim thoroughly discussed the quality considerations for 3D bioprinting products, including considerations for non-clinical testing and safety testing of the materials (e.g., toxicity, tumorigenicity). Each component of the product, especially the cell(s) and the scaffold(s), should be considered and tested independently and as a cell-scaffold composite in appropriate *in vitro* or *in vivo* preclinical models.

Professor Kim then described the clinical performance of 3D bioprinting products, with an emphasis on the engineered trachea. The trachea consists of a firm and highly structured composite of cartilage and respiratory mucosal epithelium cells, which line the inside of the trachea. He presented a new hydrogel-based bioprinting system that incorporates nasal turbinate stem cells and nasal septal chondrocytes and has been successful in biomechanical and large animal studies. In South Korea, a human clinical trial has obtained institutional review board approval, and an investigational new drug application has been submitted.

Challenges of developing induced pluripotent stem cell (iPSC)-derived cardiomyocyte patches

Assistant Professor Masao Sasai (Osaka University) shared his experience with and perspectives on the quality considerations for the manufacture of iPSC-derived cardiomyocyte patches for the repair of ischemic cardiomyopathy. When the heart has been damaged by ischemia, surgically attaching cultured myocyte cells can support heart function and accelerate healing and repair. Given the prevalence of ischemic cardiomyopathy events, there is an unmet need for treatments for ischemic cardiomyopathy, and this product may be one of the treatment options.

The QTPP for cardiomyocyte patches requires high control of the percentage of cardiomyocyte cells, their ability to hold shape (a patch), and the absence of undifferentiated iPSC, which can be tumorigenic. From an iPSC cell bank, the manufacture of cryopreserved cardiomyocytes can be performed in 30 days. The patch is then manufactured within a few days, depending on the day of administration. The CQAs of iPSC tropism, cardiomyocyte count, and the elimination of undifferentiated iPSC from the product could be met, and the product was found to be effective in animal models. In a human clinical trial, no tumors were observed, and the patch was shown to increase the contraction rate of the heart [2]. To pave the way for future clinical use, shipping stability studies have been done and they confirmed that international shipping can be accommodated.

SESSION ON PRECLINICAL SAFETY STUDIES

In the last session of the workshop, presentations on the assessment of cell therapy safety in pre-clinical settings were coordinated by the moderator, Dr Mehrshid Alai-Safar (VP, Global Regulatory CMC, Kite Pharmaceuticals). Speakers from Korea and Japan discussed their perspectives and recommendations for cell

therapy biodistribution assessment, the use of organoids in pharmaceutical evaluation, and considerations for genomic stability and tumorigenicity risk assessment.

Biodistribution of cell therapy products

Dr Yoshiteru Kamiyama (Head of Applied Drug Metabolism & Pharmacokinetics, Astellas Pharma Inc.) introduced the meeting participants to the principles of biodistribution analysis for cell therapy products. The appropriate biodistribution of a cell therapy contributes to the product's efficacy, as the beneficial effects of the therapy are often required in a particular tissue or organ type. Biodistribution is also a safety-related CQA, especially for undifferentiated stem cells that could be tumorigenic.

The data that Dr Kamiyama presented is based on the findings of a study organized by the Forum for Innovative Regenerative Medicine's Committee for Non-Clinical Safety Evaluation of Pluripotent Stem Cell-derived Product (FIRM-CoNCEPT). The two-step biodistribution study, Multisite Evaluation Study on Analytical Methods for Non-clinical Safety Assessment of Human-derived Regenerative Medical Products (MEASURE), was needed to better understand the mechanism of action and adverse events associated with cell therapy products. The goal is to inform regulators and align the industry around a qPCR-based standard biodistribution method.

The first step of the MEASURE project was a thorough landscape analysis of regulatory guidelines and guidance related to cell therapy biodistribution assessment, industry testing practices, and qPCR biodistribution method specifications. A common regulatory objective of biodistribution studies is the evaluation of safety and efficacy. The gaps between regulators emerge in the stated importance, the appropriate clinical development phase, the requirement for cell fate analysis, the recommendation of animal species, and the expectations for assay methods. Likewise, a landscape analysis of industry practices observed a variety of administration routes, development phases, animal species, assay methods, and the duration of *in vivo* biodistribution tracking studies. In analyzing qPCR-based assay methods, key areas of consensus were the target sequence, which is the Alu element for human cells, and the *Rbmy* gene of the Y chromosome for animal cells. A consensus was also reached for a 95% confidence interval for the limit of detection of a qPCR assay, while no common criterion was found for limit of quantitation, accuracy, precision PCR efficiency, or correlation coefficient.

Following the landscape analysis, a multisite experimental validation was performed for a qPCR-based assay of human mesenchymal stem cell (hMSC) biodistribution following injection into the tail veins of SCID mice. In the study, three out of five labs observed matrix inhibitory effects, most commonly in blood. The inter- and intra-assay precision and accuracy of a cell-based calibration curve varied across labs and was dependent on hMSC cell number. Across laboratories, there were large differences in the persistence of intravenously delivered hMSCs at the infusion site. All laboratories observed that the majority of cells had been distributed to the lung at the 1-h time point and most remained in the lung at the 4-h and 24-h time points. The biodistribution to lung tissue has implications for understanding the mechanism of action, safety, and efficacy of cell therapies.

Organoid-based evaluation methods

Dr Jongman Yoo (CEO, Organoid Sciences, Ltd) described the applications of 3D organoids in the screening, evaluation, and identification of advanced pharmaceutical products. Organoids are tissue or organ models that are grown from cultured and differentiated cells within a 3D structure. As a novel analytical tool, organoids have the potential to transform the preclinical evaluation paradigm for drug development by eliminating the need

for animal testing. In addition to aiding pharmaceutical development, organoids have many other potential use cases, including as regenerative medicines, in cosmetics testing, and in precision medicine diagnostics.

The first innovation discussed by Dr Yoo was the application of patient tumor-derived organoids to dramatically reshape the standard of care in oncology. Research has shown that patient tumor-derived organoids can very accurately predict, to a greater degree than tumor sequencing, the effectiveness of a particular chemotherapy. Colorectal, lung, and bile duct cancer were given as examples with demonstrated use of organoids as strong predictors of therapy success, in screening and evaluation of advanced pharmaceuticals. The current challenges of tumor-derived organoids in the evaluation of therapies with complex mechanisms of action, such as checkpoint inhibitors, were also discussed. The simplicity of tumor-derived organoids, which have a single-cell type and single-layer structure, limits their applicability to these classes of therapy.

In infectious diseases research, organoids are exceptionally useful as an alternative to animal models because infectious agents have species-specific mechanisms of action. For instance, human histo-blood group antigen expression is required for replication of human norovirus in enterocytes. Traditionally cultured cells do not express enough antigen to allow norovirus replication, but human stem cell-derived organoid systems can express sufficient antigen to model infection. Dr Yoo provided examples illustrating the utility of different organoid systems in COVID-19 and norovirus models.

The final applications presented were complex epidermal monolayer and multi-layer models of the gut and skin. These models prove to be the best tissue-specific *in vitro* models for the efficacy/pharmacokinetics evaluation of pharmaceutical compounds and for the assessment of human tissue responses. Applications and limitations in functional foods and cosmetics were also discussed.

Genomic stability and tumorigenicity

Dr Yoji Sato (Head, Division of Drugs, NIHS) presented a framework and highly performative methods for assessing the frequency and clinical significance of small numbers of potentially tumorigenic cells in a product.

To manufacture a stem cell-based product, an initial population of cells are often genetically manipulated (e.g., lentiviral transgene integration, genome editing), greatly expanded in number, and then differentiated into the therapeutic cell type, Dr Sato explained. Each of these manufacturing steps introduces tumorigenicity risk, through either increased genomic instability, the selection for genetic and epigenetic variants of oncogenes, or residual undifferentiated stem cells that have retained potentially tumorigenic stemness.

Dr Sato presented a digital soft agar growth assay with a sensitivity of 1 in 10 million that can be used to evaluate a product for the presence of transformed cells. For the analysis of residual undifferentiated pluripotent stem cells, he presented a version of the 'highly efficient culture (HEC)' assay that has improved sensitivity due to the incorporation of a magnetic-activated cell sorting step. The improved HEC assay has been validated through MEASURE (introduced by Dr Kamiyama) and described by the Health and Environmental Sciences Institute's Cell Therapy: Tracking, Circulation and Safety Technical Committee in the journal *Regenerative Medicine* [3].

The final consideration that Dr Sato discussed was the poorly understood relationship between genomic mutations and tumorigenicity. It is well known that genetic mutations are naturally incorporated into cells over time and that even healthy human beings have a mosaic of inter-cellular genetic diversity. To be able to make clinical decisions based on measurements of genomic mutations, which potentially lead to genomic instability in cell therapy products, their correlations with tumorigenic risk must be established. Dr Sato presented some of the conclusions of a 5-year

study led by Dr Shin Kawamata that extensively characterized the genomic stability of a great variety of clinically relevant cells and their potential to form abnormal tissue in immunodeficient mice [4]. One of the most predictive measures that was found is copy number variants, which predicted 86% of abnormal tissue formation.

SUMMARY AND WAY FORWARD

The *2023 NIFDS-PMDA-USP Workshop for Advanced Therapies* brought together regulatory agencies, industry stakeholders, and standard-setting organizations to share best practices and address the greatest challenges in multi-country market approval. Participants expressed a shared objective of increasing market access to the over 100 efficacious and safe Advanced Therapies that have been approved for clinical use across all markets [5]. These dialogues are essential to reach an understanding of the most important safety, efficacy, and regulatory considerations and to identify solutions to shared obstacles.

Attendees appreciated the information and perspectives on topics selected by the co-organizers as being pertinent to evolving regulatory expectations. The speakers included pioneering developers and research leaders working in Japan, South Korea, and elsewhere. Speakers shared their experiences, best practice recommendations, and perspectives on simultaneous and individual multi-national market applications. Beyond the general good practices for the development of products for international markets, speakers highlighted specific regulatory expectations that are unique to each region. By keeping these unique expectations in mind, developers and agencies can find ways to increase the availability of these life-changing therapies.

In closing the workshop, Dr Soo Jung Sohn, Director General of PMDR, NIFDS, emphasized that advanced therapy clinical trials are growing in number, over 6000 at the beginning of 2024 [4], and are on pace to continue this growth. To ensure that these therapies reach the patients who need them, it will be essential to maintain, and even bolster, inter-agency and stakeholder-agency dialogue and collaboration, which were highlighted on day 1 of the workshop [1]. The workshop's participating organizations, NIFDS, PMDA, and *USP* will develop regulations, guidance, and internationally recognized reference standards that are informed by the discussions of the 2-day workshop. They will also convene future events for advanced therapies stakeholders to discuss existing and emerging challenges. Through the collaborative efforts of industry, academia, and regulatory agencies, the promising future of advanced therapies will become an accessible and transformative reality.

REFERENCES

1. Minkyung Kim M, Clarke B, Chase CG, *et al.* 2023 NIFDS-PMDA-USP Workshop for Advanced Therapies, day 1. *Cell Gene Ther. Insights* 2024; 10(5), 653–658.
2. Miyagawa S, Kainuma S, Kawamura T, *et al.* Case report: transplantation of human induced pluripotent stem cell-derived cardiomyocyte patches for ischemic cardiomyopathy. *Front. Cardiovasc. Med.* 2022; 9.
3. Watanabe T, Yasuda S, Chen CL, *et al.* International evaluation study of a highly efficient culture assay for detection of residual human pluripotent stem cells in cell therapies. *Reg. Med.* 2023; 18(3), 219–227.
4. Yamamoto T, Sato Y, Yasuda S, *et al.* Correlation between genetic abnormalities in induced pluripotent stem cell-derivatives and abnormal tissue formation in tumorigenicity tests. *Stem Cells Transl. Med.* 2022; 11(5), 527–538.
5. American Society of Gene and Cell Therapy, Citeline. *Gene, Cell, and RNA Therapy Landscape Report Q1 2024 Quarterly Data Report*. 2024; American Society of Gene and Cell Therapy, Citeline. https://asgct.org/global/documents/asgct-citeline-q1-2024-report.aspx.

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Accelerating process analytics: leveraging dPCR to quantitate AAV viral titers

Viki Mahendran, Scientist III, Molecular Biology, Thermo Fisher Scientific

In cell and gene therapy manufacturing, accurately detecting AAV genomes is vital to help ensure the efficacy and safety of the final product. Digital PCR (dPCR) is a highly sensitive and precise technique that enables the absolute quantification of nucleic acid targets. In this poster, the Applied Biosystems™ ViralSEQ™ dPCR AAV Titer Kit is assessed for its effectiveness in calculating viral titer using both extraction and extraction-free protocols.

dPCR provides a robust and accurate method for measuring viral titer, a critical parameter in assessing the purity and potency of biopharmaceutical products. The Applied Biosystems ViralSEQ dPCR AAV Titer Kit can detect both ITR2 and SV40 within the AAV genome and can be used to accurately assess viral titer in both extracted and extraction-free samples.

Table 1. Assay performance specifications of the Applied Biosystems™ ViralSEQ™ dPCR AAV titer kit

Assay performance	AAV
Linearity	R2>0.99
Precision at LOQ	≤20% CV
LOD	9 copies/rxn
LOQ	27 copies/rxn (ITR2) 27 copies/rxn (SV40)
Assay range	9–90,000 copies/rxn

CV: cell volume; LOD: limit of detection; LOQ: limit of quantification; rxn: reaction.

EXTRACTION-FREE VERSUS EXTRACTION SAMPLE PREPARATION

The application and suitability to use either extraction or extraction-free samples is dependent on the type of sample. Extraction-free preparation is simpler and minimizes processing time and sample losses; however, the method has limitations, as the presence of inhibitors may interfere with the accuracy of results. Sample extraction kits are effective in removing inhibitors, allowing for accurate quantification of complex samples, although the extraction process increases processing time and there may be losses, leading a reduction in the quantity of detectable viral particles.

VIRALSEQ dPCR AAV TITER KIT PERFORMANCE

The performance of the dPCR AAV kit has been optimized and validated on the Applied Biosystems™ QuantStudio™ Absolute Q™ dPCR system. Meeting the specifications outlined in Table 1, the kit enables end users to accurately assess AAV viral titers. To assess AAV8 viral titer, two protocols using the dPCR kit were compared: one utilizing extracted samples with the Applied Biosystems™ KingFisher™ sample preparation method and the other applying an extraction-free protocol.

Upon completion of these runs, the QuantStudio Absolute Q dPCR system processed the data into 1D plots, as shown in Figure 1. In this experiment, the internal positive control results were similar to the no template control (NTC), indicating that inhibition was not present. Distinct separation between the positive and negative populations was observed for both targets. As anticipated, there were differences in the concentration readouts of AAV8 obtained from the two sample extraction methods. In order to determine the true stock concentration, both readouts need to be recalculated using ITR2 target, considering any dilutions that may have occurred during the extraction process.

RESULTS & ANALYSIS

From these calculations, the following results were found:

For extraction-free samples:

- If the ITR2 concentration from the serial dilution is 795 copies/µL, sample input is 3 µL into 9 µL reaction volume per well; the DNase dilution is 20, and the accumulated sample dilution is 10,000, then:

- 795 cp/µL × (9/3) × 20 × 10000 × 1000 = 4.77 × 1011 copies/mL

- 100-fold dilutions were carried out twice from DNase-treated samples

For extracted samples:

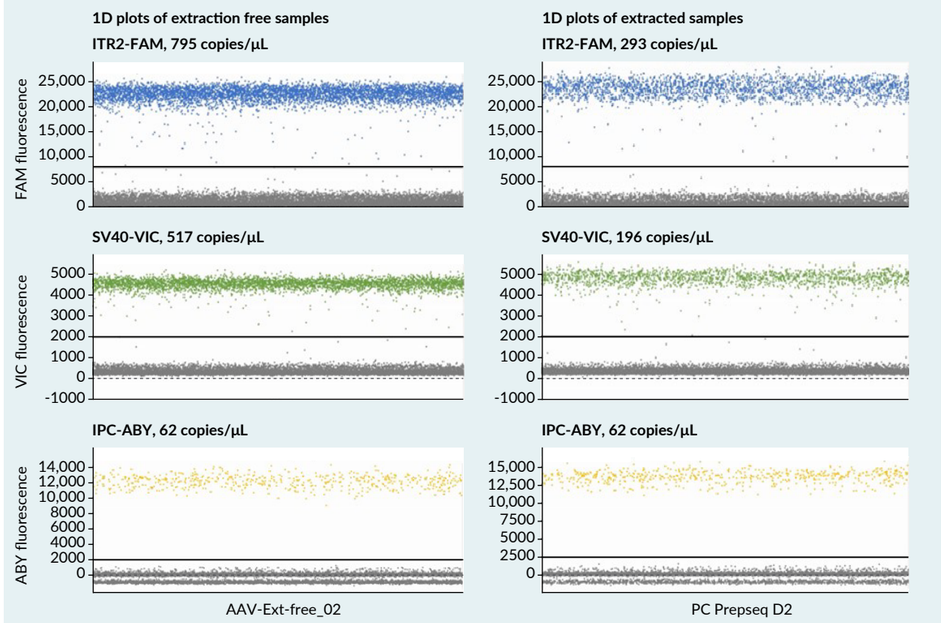
- If ITR2 concentration from the serial dilution is 293 copies/µL, sample input is 3 µL into 9 µL reaction volume per well; the DNase dilution is 20, PrepSEQ method is 2, and the accumulated sample dilution is 1000, then:

- 293 cp/µL × (9/3) × 20 × 2 × 1,000 × 1,000 = 3.52 × 1011 copies/mL

- 100-fold dilutions were carried out twice from DNase-treated samples

The results indicate that direct dPCR using the extraction-free method detected higher levels of AAV8 compared to extracted samples, implying potential losses during the purification process. This suggests that direct dPCR can be more efficient and accurate for quantifying AAV8 when sample integrity is critical. However, for matrices containing components that might inhibit dPCR, it is advisable to perform sample extraction before dPCR analysis to facilitate accuracy and reliability.

Figure 1. Detection of ITR2 and SV40 in extraction-free and extracted samples.



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In partnership with: ThermoFisher SCIENTIFIC

CELL & GENE THERAPY INSIGHTS

GENE THERAPY ANALYTICS AND CMC

SPOTLIGHT

INTERVIEW

Navigating gene therapy QC: exploring the expanding AAV vector analytical toolkit

Abi Pinchbeck, Editor, *Cell & Gene Therapy Insights*, speaks to **Van Hoang**, Head of Analytical and QC, Spark Therapeutics, exploring three key challenges in the field of AAV vector analytics and QC and how the expanding analytical toolkit can enable solutions to be found. They also discuss the development of novel technologies to gain a deeper understanding of the empty, full, and partially filled capsid analysis picture.

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Q What are you working on right now?

VH: At Spark, we are on a mission to unlock the power of gene therapy to accelerate healthcare transformation and change lives for patients, families, and communities everywhere. Right now, I am excited about SPK-8011, our investigational gene therapy for patients with severe or moderately severe hemophilia A. SPK-8011 is a novel bioengineered AAV vector that uses the AAV-LK03 capsid, or Spark200 capsid, which contains a codon-optimized human factor VIII (FVIII) gene under the control of a liver-specific promoter. It is a one-time

"As an industry, we need to find a way to reduce the volume needed for testing, as innovation here would be transformative for the field."

gene therapy option, that aims to lessen the burden of a lifetime treatment that current hemophilia A treatment options require. Spark is now enrolling for the Phase 3 trial that aims to produce durable FVIII expression within a predictable therapeutic range, using a low dose, coupled with a standardized immunomodulatory regimen that is safe and effective.

Additionally, since its approval in 2017, we have worked hard to ensure that Luxturna™ is available to patients, and we remain committed to consistently supplying this important gene therapy worldwide.

Q How would you describe the current field of AAV vector analytics and QC? What are the key challenges and questions the field is facing now?

VH: There are a few industry-wide challenges. The first is related to product yield and analytical testing, namely the vector material consumption required for testing to release products to clinical trials or the commercial supply chain. The large volumes required for testing can dramatically impact the supplies available for patients.

As an industry, we need to find a way to reduce the volume needed for testing, as innovation here would be transformative for the field. We need equipment vendors to continue working on technologies in this area, such as miniaturization to reduce volumes needed for testing, and ways to multiplex or establish multi-attribute assays that use less material overall and give multiple readouts on quality attributes from one testing instance. These things are needed to support process development, drug substance and drug product development, and the product release space.

Another hot topic is potency assays. These assays are incredibly important in advancing a product, and developing potency assays can be highly challenging, especially for products with a complex mechanism of action, which is many of them. This includes mechanisms of action that may not have been fully validated, or that involve a cascade of events. Developing an assay that reflects a complex cascade of events can be challenging.

Another industry-wide challenge is the number of available representative lots early in development. We have a limited number of representative lots, which can make setting acceptance criteria for product specifications challenging early on in development. The availability of representative lots compounds the challenge around potency assays. A developer with a limited number of representative lots available for developing an assay may not be able to fully establish and understand assay performance.

Q How can the field best leverage the expanding analytical toolkit for viral vector characterization and QC?

VH: One challenge lies in accelerating our ability to get products to patients more quickly. Some of this acceleration can be done by leveraging existing methods in the analytical toolkit and developing a platform approach to the way we do things.

The field is now ready to enable the implementation of platform approaches to development, including to processes, or phase-appropriate platform approaches to analytical strategy. In our case, we are implementing a platform analytical strategy that consists of a set of attributes and methods at the appropriate phase for both product development and product testing. This great opportunity will help us streamline the way we work, increasing efficiency and enabling acceleration.

Q How do you see the empty, full, and partially filled capsid analysis picture continue to develop?

VH: Empty-full is one of the more complex topics in the field right now. Fundamentally, the *in vivo* impact of empties is not well established or fully known. We are seeing regulatory expectations evolve towards a continual reduction in the number of permitted empties in the product, and there has been progress in process development to ensure this is the case.

Specifically, from the analytical and quality control perspective, the available analytical toolbox is comprehensive, with technologies such as cryogenic electron microscopy, charge-detection mass spectrometry, analytical ultracentrifugation, size-exclusion chromatography with multi-angle light scattering, and many more. Our ability to build a detailed understanding of the distribution of empty, full, and partially filled capsids has evolved well over the last 5 years. There are many tools available to developers, whether in-house, or through the use of contract testing labs.

Another important consideration for the picture of empty, full, and partials is coupling these methods with fractionation experiments and other extended characterization methods such as next generation sequencing or other assays to understand the capsid profile. Incorporating these characterization methods during development will provide a more detailed picture of product quality.

Q What impact are instruments capable of multiple in-process assays having on cost and efficiency? Which analytical innovations stand out for you?

VH: There is still a strong need for technology that can do many of the things that we want. There is a need for equipment that can be used for in-process measurements with

"We are working hard on the Phase 3 trial for SPK-8011, which is a top priority."

multi-attribute readouts. With regard to multi-attribute readouts, equipment that could from a single sensitive measurement online, provide data for titer, empty/full/partially filled, and aggregates would be highly useful.

The technology is not quite there yet, however, we are all committed to exploring the space further. There remains a strong need for online sensitive measurements for titer, empty/full, and better measurements for aggregates.

Q As developers look to consider recent key guidances, where is further guidance and standardization most needed?

VH: Overall, global harmonization continues to pose a challenge. Greater consistency of review across health authorities and the standardization of guidances will help to streamline the review process and enable developers to quickly progress a phase-appropriate platform analytical strategy. Global convergence and harmonization of health authority expectations would benefit developers of AAV-based therapies.

Q What are your priorities for yourself and for your team at Spark over the next few years?

VH: We are committed to developing an approach to patient access tailored to the unique nature of an investigational, potentially one-time, life-altering gene therapy. We are working hard on the Phase 3 trial for SPK-8011, which is a top priority. This is an important investigational gene therapy with the potential to change lives. We are also hopeful to advance further findings in the areas of systemic central nervous system delivery and ocular gene therapy.

BIOGRAPHY

VAN HOANG has over 20 years of experience leading analytical organizations and developing medicines for patients globally. Her area of expertise is in development of gene therapies, vaccines, and therapeutic proteins. Van joined Spark Therapeutics, Philadelphia, PA, USA in March 2020. She currently serves as Head of Analytical and Quality Control at Spark Therapeutics, where she leads an organization focused on analytical development, quality

control testing, and operations for assets spanning preclinical development through marketed products including asset lifecycle maintenance. Before joining Spark Therapeutics in March 2020, Van was at Merck for 17 years. Van holds a PhD in Pharmaceutical Chemistry from University of California San Francisco, San Francisco, CA, USA and a BSc in Biochemistry from the UCLA, Los Angeles, CA, USA, graduating with *magna cum laude* honors.

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CELL & GENE THERAPY INSIGHTS

INTERVIEW

Exploring analytical assays and CMC challenges for viral vector-based therapies

Abi Pinchbeck, Editor, *Cell & Gene Therapy Insights*, speaks to **Vladimir Slepushkin**, Chief Technology Officer, MedTherapy Biotechnology, to explore the state of the art and novel tools in lentiviral and AAV analytics and CMC. They discuss the delicate interconnectivity between the upstream and downstream, the need for global regulatory harmonization for advanced therapies, and the importance of developing processes and assays simultaneously.

Cell & Gene Therapy Insights 2024; 10(6), 729–733

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Q What are you working on right now?

VS: MedTherapy Biotechnology is a startup that manufactures lentiviral and retroviral vectors and CAR-T cell therapies. Our main goal is to make cell and gene therapies more affordable for people in developing countries like India, so we have established a GMP manufacturing facility near New Delhi.

"The main problem that the field of AAV is facing is the immunogenicity of this vector."

Q How would you sum up the current status of the analytical toolkit for both lentiviral and AAV vector production? Where did the key challenges and gaps remain?

VS: The toolkit for both AAV and lentiviral vectors is quite developed at this stage, without many remaining gaps. There are however some difficulties based on the nature of these therapies. AAV is almost exclusively a directly injectable vector, which works *in vivo*. The main problem that the field of AAV is facing is the immunogenicity of this vector. Immune responses to AAV can cause some adverse events and even death in some extreme cases. This challenge underlies the need to balance the ratio of empty to full viral particles–empty capsids must be minimized to reduce immunogenicity.

Lentiviral vectors are the field in which I am most involved. The challenges here are completely different because lentiviral vectors are almost exclusively used for the transduction of cells *in vitro*. This creates a different set of problems, mostly related to genotoxicity due to the integration of the vector inside the genome of the cells. The vector can potentially integrate in parts of the genome containing oncogenes, thereby inducing oncogenesis and potentially leading to tumors or cancer in patients. That being said, the risks of lentiviral vectors are much lower than those for retroviral vectors, which were used previously for this purpose.

In regards to analytical assays, it must be proven that the lentiviral vector does not integrate near oncogenes. This requires methods such as next-generation sequencing (NGS) and insertion site analysis, which are techniques that require a lot of effort, time, and money. Potency assays are relevant to both vector types, and these potency assays must be developed and individualized for each target or gene of interest, meaning that they are relatively difficult to develop. Fortunately, the US FDA recently published guidance specifically for potency assays to help people overcome this.

Q How can we aim to overcome the existing difficulties in vector analytics and gene therapies?

VS: Due to new technological developments, we have better tools than ever to assess all the required parameters. One of these tools is NGS, which is being used increasingly widely in analytics to characterize vectors and transduced cells.

The other notable technological development is a new generation of flow cytometry and single-cell analysis. This is most relevant to lentiviral vectors. Within potency assays, it can be

difficult to understand the cells and mechanisms of action that are involved to identify what is important to measure. New assays such as new flow cytometry assays, such as CyTOF, or single-cell analysis could assist in that.

Q How do you collaborate with cross-functional teams, including research, manufacturing, and regulatory affairs to ensure seamless development of gene therapy products?

VS: Developing a gene therapy product starts with R&D. At this early stage, it is critical to develop your processes and assays simultaneously. You cannot do one without another, as they are connected in more than one way. Often, people pay much more attention to process development and manufacturing than to assay development, which is an essential part of overall development.

Collaboration between analytical and process development departments is highly important. The process development stage undergoes tech transfer into GMP manufacturing, and assay development into quality control. Quality oversight is required over all these stages, and QA is very important.

Then, regulatory assessment is needed to ensure that the assays and manufacturing procedures are compliant with current regulations. Unfortunately, those regulations are different in different geographies, for example, there are many differences between US, Indian, Japanese, and European regulations. The regulatory assessment stage is critical in guiding the manufacturing and quality control to be acceptable in each geography.

Q In lentiviral manufacturing, what downstream challenges are thrown up by efforts to both boost upstream productivity and how can these challenges be addressed?

VS: The downstream problem for lentiviral vectors is that they are quite fragile and tend to become inactivated during downstream processing. They do not tolerate any temperature or pH changes, and they also tend to aggregate. If they do aggregate, the final sterile filtration through 0.22 µm filters will be challenging as they will not go through the filter and you will lose your product.

The main challenge there is to develop a downstream process that still gives you high yields, whilst also maintaining good quality in terms of purity. Lentiviral vectors are not injected directly, giving some leeway with purity as some impurities can be removed at the *in vitro* transduction stage during cell washes, for example. Nevertheless, the vector must be pure enough to allow good transduction efficiency for the cells—impurities often inhibit vector activity leading to problems with transduction.

"It would be highly advantageous for the space if regulatory agencies globally were in agreement."

Regarding the connection with upstream, the biggest thing people are developing now in this area is the use of suspension cell culture instead of the adherent culture we used previously. The main advantage of that for downstream is that bovine serum is not needed to produce the vectors when using suspension cultures. This means that the upstream product/downstream starting material is much purer than when using adherent culture. That facilitates and improves yields downstream.

Q Can you dissect the key challenges in the CMC regulatory space for vector-based therapies, and what do you see as future trends here?

VS: One of the main challenges is that there are different regulations for different geographies. Regulatory agencies are trying to resolve this and harmonize regulations, with specialized harmonization bodies aiming to standardize global regulations, but for now, unfortunately, many differences remain. It would be highly advantageous for the space if regulatory agencies globally were in agreement.

In addition, potency assays are difficult to prove to regulatory agencies, because in addition to showing that your product works in general, you must also quantify the assay. This can pose issues, due to some variabilities in biological assays. This requires some creativity, especially if you do not precisely know the action mechanism.

BIOGRAPHY

VLADIMIR SLEPUSHKIN is Chief Technology Officer at MedTherapy Biotechnology, Boston, MA, USA. He leads all functions associated with manufacturing of CAR-T cells and viral vectors. Previously he was Executive Director of Vector Technology at Autolus Therapeutics, leading process development for manufacturing of lentivirus vectors in suspension cell culture, guiding assay development to support process development for lentiviral vectors, and managing CMO for GMP vector production and T-cell processing. Before that, Dr Slepushkin was directing research vector core, and providing lentiviral, retroviral, and AAV vectors for Kite Pharma. Vladimir proved successful in developing novel high-quality products by managing diverse technical groups and cross-functional teams, developing first-in-class clinical product from scratch, including facilities, equipment, manufacturing process, quality systems, regulatory CMC submissions, and clinical trials design. He has proven expertise in technically understanding and leading the development and improvement of cell culture and purification processes, and operations and analytical methods, adhering to customer, regulatory, safety and environmental requirements, and guidelines. Vladimir is experienced in identifying and resolving regulatory and manufacturing technical problems, as well as

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CELL & GENE THERAPY INSIGHTS

GENE THERAPY ANALYTICS AND CMC

SPOTLIGHT

INTERVIEW

Advancing the analytical toolkit to drive viral gene delivery

Bristol Myers Squibb's **Chelsea Amstuz**, Associate Director, Vector Attribute Sciences, and **Itzcoatl Pla**, Executive Director, Gene Delivery and Editing Process Development, highlight the current state-of-the-art in analytical innovation for gene delivery, including considerations for both viral and non-viral delivery.

Cell & Gene Therapy Insights 2024; 10(6), 735–738

DOI: 10.18609/cgti.2024.088

Q What are you working on right now?

Currently, our team is focused on gaining a comprehensive understanding of viral vector biology and identifying the key attributes that determine the suitability of a viral vector for use in cell therapy.

"Next-generation sequencing, including whole genome sequencing, can be leveraged to gain insights into AAV quality and gene editing."

Q What do you see as the current key challenges/hurdles in the field of analytics for gene delivery, considering lentiviral vectors, AAV, electroporation, and LNPs?

In the field of analytics for gene delivery, we face several challenges across lentiviral vectors, AAV, electroporation, and LNPs. One major challenge is the lack of efficient tools for quickly assessing the quantity and quality of lentiviral vectors during manufacturing. Existing assays take anywhere from 8 hours to 7 days to provide useful information. While AAV has more options for titer measurement, it requires high concentration and purity. We need innovative technologies that enable rapid vector titer measurements, even in low-concentration or impure samples. Additionally, measuring vector quantity alone is not sufficient; ratios of multiple vector attributes, such as total particles to infectious particles or transduction potential, provide more informative insights.

Q How can we best tackle these challenges? How can the key areas of need for (affordable) analytical innovation be addressed?

Tackling these challenges requires collaboration and open communication. While one expert may not have all the answers, it is important to share the key challenges with internal and external partners who may have innovative solutions. By fostering partnerships, we can collectively address the areas of need for affordable analytical innovation.

Q How can the field best leverage the expanding analytical toolkit for viral vector characterization and QC, given the increasing complexity of novel vectors?

With the increasing complexity of novel vectors, the expanding analytical toolkit offers valuable opportunities for viral vector characterization and quality control. Next-generation sequencing, including whole genome sequencing, can be leveraged to gain insights into AAV quality and gene editing. By harnessing these advanced analytical techniques, we can enhance our understanding of viral vectors and optimize their application in cell therapy.

Q How will the empty-full-partially full capsid analysis picture continue to develop in terms of both regulatory guidance and choice of analytical technology options?

The analysis of empty-full-partially full capsids is of utmost importance in our field. For AAV, there are several analytical tools available to measure the empty-full ratio. There are traditional methods like analytical ultracentrifugation, chromatography assays, and the ratio of capsid protein to genomes, in addition to novel technologies like mass photometry. The challenge lies in selecting the most appropriate tool, as each option has its own advantages and limitations. In the past, it has been challenging to measure the proportion of empty lentiviral particles. New tools like the Leprechaun have recently become commercially available, and we are actively evaluating their potential to address this measurement challenge.

Q What are your goals and priorities in both your own work and for Bristol Myers Squibb over the next 1–2 years?

Over the next few years, our primary goal is to gain a comprehensive understanding of viral vector attributes that drive efficient transduction and gene editing or otherwise make a viral vector favorable for use in cell therapy. As a company, our focus in cell therapy is to reduce our turnaround time and reduce our overall out-of-specification rate, to further strengthen our ability to supply to our patients.

BIOGRAPHIES

CHELSEA AMSTUZ has 14 years of molecular virology experience and has worked in the cell and gene therapy space for 8 years, serving as both a virology subject matter expert and leading analytical development and operations teams. She has extensive experience in molecular, cellular, and biophysical assay development, especially in the context of viral vector characterization. Chelsea currently leads Vector Attribute Sciences at Bristol Myers Squibb, Seattle, WA, USA. Chelsea has a PhD in Cellular and Molecular Biology from the University of Washington, Seattle, WA, USA and a BSc from Emory University in Atlanta, GA, USA.

ITZCOATL PLA has more than 20 years of experience in the biopharmaceutical industry, ranging from process control, process development, process characterization, large-scale clinical and commercial manufacturing, establishing new experimental and technical teams, transferring processes to and from third-party manufacturers, process validation, and life-cycle management and licensure by various health authorities. Itzcoatl currently leads the Gene Delivery and Editing, Process Development team in Cell Therapy at Bristol Myers Squibb, Summit West, NJ, USA. Itzcoatl joined Bristol-Myers Squibb in July 2013 as the Director for Biologics MS&T Drug Substance, Global Capabilities, then Executive Director of Parenteral MS&T, and later Head of Viral Vector Internal and External Clinical Manufacturing, Process and Analytical Development and MS&T. Itzcoatl previously worked for Abbott Laboratories and Abbvie. Itzcoatl holds a BS in Biochemical Engineering from

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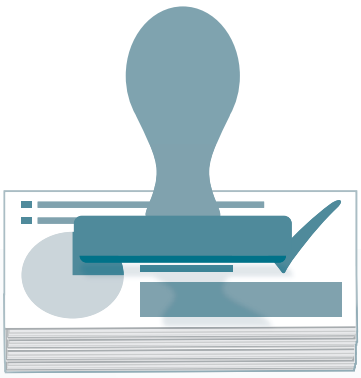

CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Advancing AAV production with high-throughput screening and transcriptomics

Christopher A Reid, Markus Hörer, and Mohammad A Mandegar

AAV is a widely used vector for *in vivo* gene delivery that often requires considerable manufacturing capacity. However, current manufacturing techniques are inefficient, leading to high production costs and product impurities that could limit efficacy and risk patient safety. These factors severely limit the widespread use of AAV-mediated gene therapies for common and rare indications. To develop, manufacture, and commercialize AAV products more efficiently and cost effectively, the manufacturing process needs improvements. Herein, we transcend traditional strategies for developing DoE processes by sharing a new integrated approach that combines high-throughput screening and transcriptomics. This approach can yield proprietary datasets and insight into the mechanisms of AAV production. We also outline how discoveries and innovations in upstream production can be improved to amplify the product yield and quality over the next decade. These efforts will enable the field to fully realize the commercial and therapeutic promise of AAV gene therapies.

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AAV-BASED GENE THERAPY HAS EMERGED AS A MAINSTAY OF MODERN MEDICINE

AAVs are nonpathogenic dependoparvoviruses that contain a single-stranded DNA genome of approximately 4.7 kb [1,2]. Recombinant AAVs have gained popularity in the gene therapy field due to their well-established safety profile, broad and tunable tropism, nonpathogenic nature, and capability of achieving long-term expression in non-dividing cells with a single treatment. Also, with capsid engineering, the tropism of these vectors can be enhanced to increase the efficiency of transduction of the desired tissue

type, limit the host immune response, and de-target from undesired tissues to improve safety [3,4]. As of 2024, AAV gene therapies have been administered to more than 3000 patients, and more than 700 programs are currently being developed (Figure 1) [5–9]. These programs focus on a range of indications, including neurologic, ophthalmologic, metabolic, neuromuscular, and cardiovascular diseases. They also use a variety of AAV serotypes. The four most common AAV serotypes used in clinical trials and approved as gene therapies are AAV9, AAV8, AAV2, and AAV5 (Figure 1). As a gene-delivery vehicle, AAV is generally safe and well-tolerated at doses below 1×10^{14} vg/kg [5,10]. However, doses greater than 1×10^{14} vg/kg need more careful monitoring in patients to assess potential safety concerns in the context of the disease status [11].

As of June 2024, 7 AAV gene therapy products are commercially available and approved by either the European Medicines Agency (EMA) or the US FDA (Table 1). In 2012, Glybera® became the first approved AAV gene therapy by the EMA for treating lipoprotein lipase deficiency. However, 5 years later, Glybera was withdrawn from the market due to commercial considerations [12]. Current approved AAV products include Luxturna® for Leber's congenital amaurosis, a rare inherited retinal dystrophy; Zolgensma® for spinal muscular atrophy type 1; Upstaza™ for aromatic L-amino acid decarboxylase deficiency; Hemgenix® and Beqvez™ for hemophilia B; Roctavian™ for hemophilia A; and Elevidys® for Duchenne muscular dystrophy [4,13]. Several more therapies are in late-stage clinical trials, and the FDA predicts that 10–20 cell and gene therapy products may be approved by 2025 [14].

CURRENT AAV MANUFACTURING PRACTICES

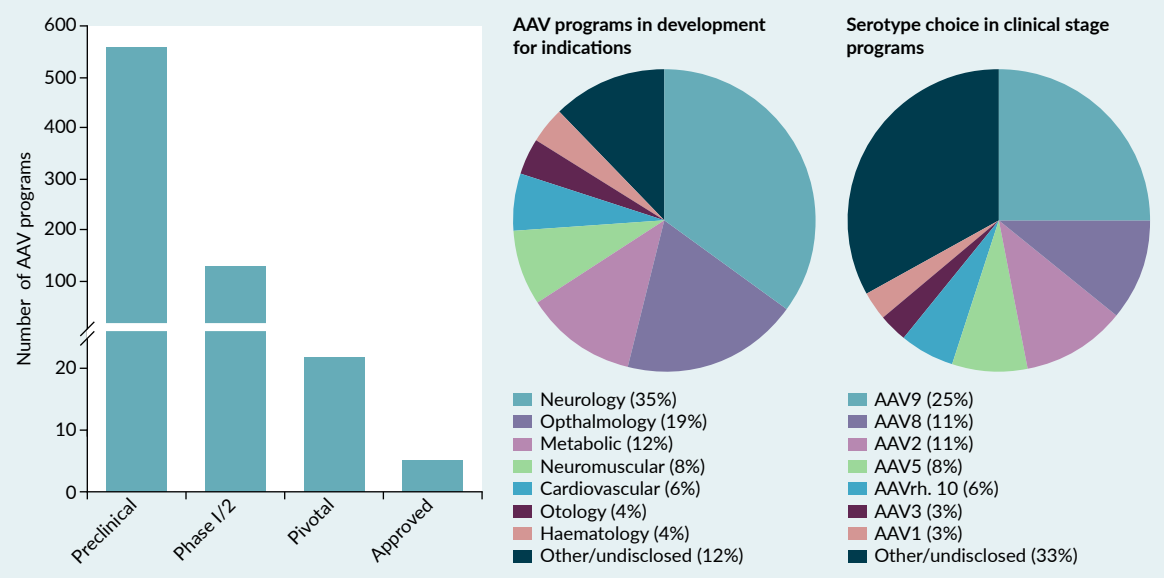
Existing AAV production platforms rely on cells for manufacturing. The most common AAV manufacturing platform uses mammalian cells, primarily human embryonic kidney 293 (HEK293) and its derivatives, and less commonly HeLa, baby hamster kidney (BHK), and Chinese hamster ovary (CHO) cells. In these platforms, genetic material needed for recombinant AAV (rAAV) production is delivered through plasmid transfection [15–20] or viral transduction [17,21,22]. Alternatively, non-mammalian systems rely on *Spodoptera frugiperda* (*Sf9*) insect cells and baculovirus transduction [23,24]. Most recently, a transient plant-based system was described for rAAV production [25]. Also, stable packaging and/or producer cell lines have been used for rAAV manufacturing after induction by chemicals or helper viruses [19,26–31]. So far, five of the commercial AAV products use HEK293 cells, and two use the *Sf9*-baculovirus system (Table 1).

Several factors can influence selection of the AAV manufacturing platform, including required yields, scalability, capsid serotype, and regulatory considerations. As of 2024, the top performing *Sf9*-baculovirus systems have produced higher volumetric yields than the HEK293 system [32,33]. However, there is no clear consensus on which platform is ideal for AAV production due to variations in quality and potency of the vector produced by each system. Some of these differences may be driven by capsid serotype, cargo, and platform [32,34–36]. Although most early-stage AAV programs do not explicitly disclose which manufacturing platform is used for viral production, available public information suggests that around 85% of current AAV manufacturing uses mammalian cells [37,38]. Therefore, in this commentary, we focus on the production system that uses transient transfection of HEK293 cells.

The process of manufacturing rAAV drug product is categorized into upstream (drug substance) and downstream (drug product) processes. The upstream process defines the vector yield, integrity, infectivity, and potency. The process also describes several crucial product-derived impurities, including, but not limited to, full/empty

FIGURE 1

Landscape of AAV gene therapy programs.



More than 700 AAV programs are being developed for a range of indications, including neurologic, ophthalmologic, and metabolic conditions. The most commonly used AAV serotypes used in clinical trials include AAV9, AAV8, AAV2, and AAV5.

TABLE 1

AAV-based gene therapy products that are commercially available and approved as of 2024.

AAV therapy (company)	Indication	Vector	Manufacturing platform	Approval	Dose	Price, million $ USD (2024)
Luxturna® (Roche)	Leber congenital amaurosis	AAV2 *RPE65*	HEK293	2017 (FDA) 2019 (EMA)	1.5×10^{11} vg/eye	0.85
Zolgensma® (Novartis)	Spinal muscular atrophy	AAV9 *SMN1*	HEK293	2019 (FDA) 2020 (EMA)	1.1×10^{14} vg/kg	2.1
Upstaza™ (PTC Therapeutics)	Aromatic L-amino acid decarboxylase deficiency	AAV2 *DDC*	HEK293	2022 (EMA)	1.8×10^{11} vg	3.7
Hemgenix® (CLS Behring)	Hemophilia B	AAV5 *F9*	*Sf9*	2022 (FDA) 2023 (EMA)	2×10^{13} vg/kg	3.5
Roctavian™ (BioMarin)	Hemophilia A	AAV5 *F8*	*Sf9*	2022 (EMA) 2023 (FDA)	6×10^{13} vg/kg	2.9
Elevidys® (Sarepta Therapeutics)	Duchenne muscular dystrophy	AAVrh74 *Micro-DMD*	HEK293	2023 (FDA)	1.33×10^{14} vg/kg	3.2
Beqvez™ (Pfizer)	Hemophilia B	AAVrh74 *F9*	HEK293	2024 (FDA)	5×10^{11} vg/kg	3.5

HEK293: Human embryonic kidney 293.

capsid ratios, residual plasmid and host-cell DNA packaging, and posttranslational capsid modifications. Thus, to improve the quality, potency, and yield of the final rAAV drug product, the upstream process must be optimized. During such optimization,

typical refinement steps include producer cell–line selection, media formulation, plasmid sequence and ratio optimization, transfection reagent selection and optimization, cell expansion and scale-up, bioreactor process optimization and monitoring. The downstream process typically involves concentration of the producer cells, cell lysis, nuclease treatment, affinity or hydrophobic interaction chromatography for capture and purification, tangential flow filtration for concentration and rebuffering, ion exchange chromatography and/or ultracentrifugation polishing, and full capsid enrichment, formulation, fill, and finish (Figure 2) [39,40].

MANUFACTURING BOTTLENECKS LIMIT THE WIDESPREAD USE OF AAV GENE THERAPIES

Although rAAV-based medicines have shown long-term and robust safety and efficacy in the clinic, these treatments have prohibitive costs, particularly in the case of systemically delivered therapies [39]. For example, in 2019, Zolgensma became the most expensive medicine developed, with a price of US$2.1 million per patient for treating spinal muscular atrophy [41]. In 2022, Hemgenix and Upstaza surpassed Zolgensma as the most expensive drug, with a one-time price of US$3.5 million (Hemgenix) and US$3.7 million (Upstaza) per patient (Table 1) [42].

Another obstacle in slow adoption of rAAV gene therapies involves challenges with manufacturing. These challenges include non-scalable processes inherited from academic settings [43]; batch-to-batch variability [4]; poor quality due to low full-to-empty capsid ratios; packaging of undesired, potentially harmful DNA sequences [40,44]; capacity constraints; shortage of trained experts; and higher regulatory demands for manufacturing stringency [39,45]. Moreover, due to the relative novelty of AAV gene therapies, product developers have prioritized accelerating their medicines through clinical development and regulatory approval. This strategy often overlooks the opportunity and need to optimize manufacturing processes during the early stages of product development. These problems then persist after therapies are approved because product developers have little incentive to improve the established process that gained regulatory approval.

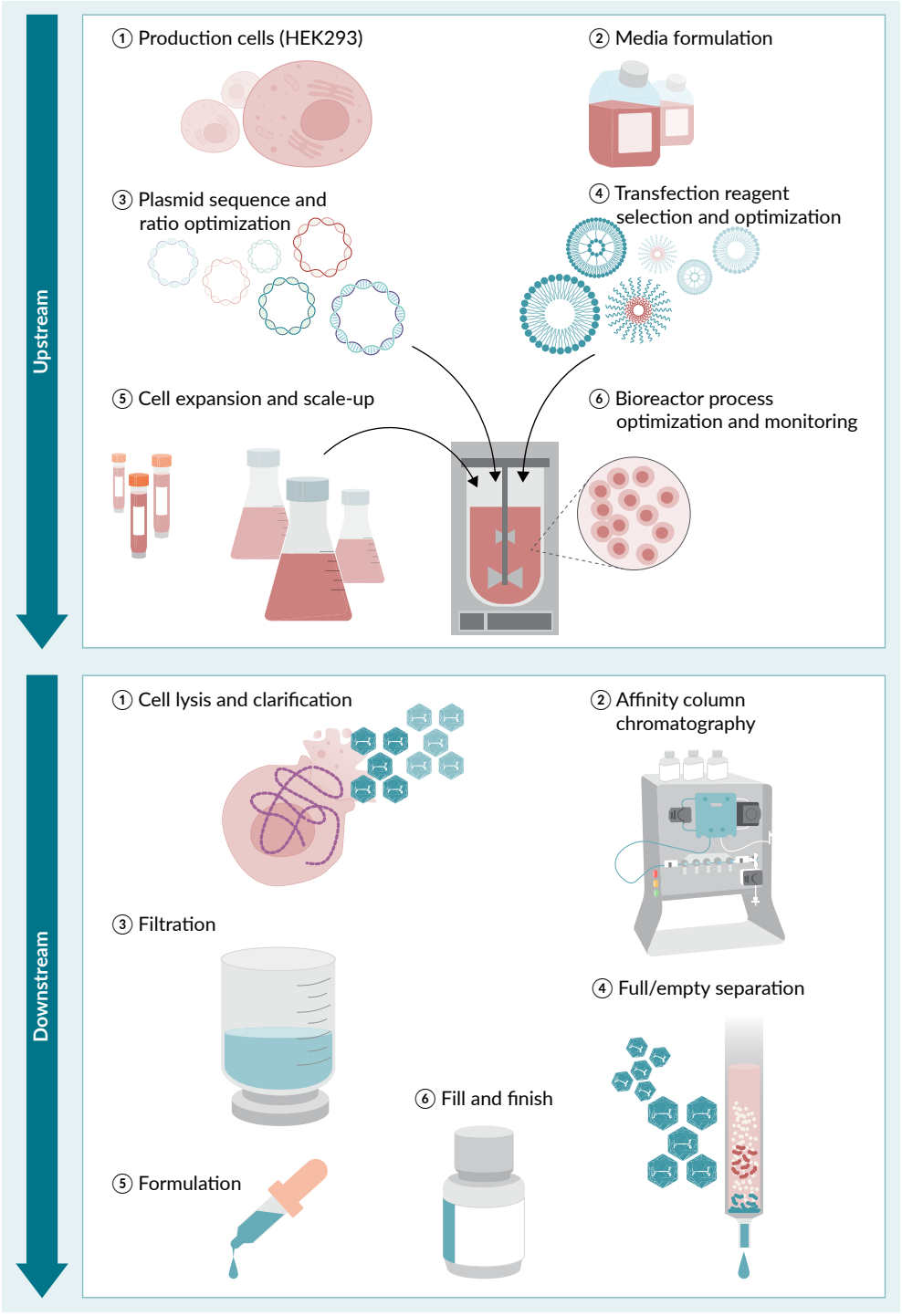
A MOVE TOWARD AAV THERAPIES FOR MORE COMMON INDICATIONS

Fortunately, the demand for viral production has not yet been great enough to strain the system. The lower demand is because rAAV-based gene therapies have mainly targeted ultra-rare and rare monogenic diseases, such as Leber's congenital amaurosis, spinal muscular atrophy, and hemophilia A and B. However, rAAV therapies are being explored for more common indications, such as Duchenne muscular dystrophy, that require a high systemic dose of vector [10]. These therapies are rapidly advancing through the clinic and will require a considerable amount of vector to keep up with patient demand [46]. Moreover, as rAAV-based gene therapies continue to build momentum, they will likely be developed to address more prevalent conditions. Indeed, preclinical programs are already targeting common diseases, such as diabetes [47], hypertension [48], Alzheimer's disease [49,50], and heart failure [51–53].

Due to the growth of AAV gene therapies and their potential to treat highly prevalent conditions, the demand for viral production will rapidly exceed the capacity of manufacturers if processes are not improved (Figure 3). Currently, most systemically delivered AAV gene therapies require a total dose ranging from 1×10^{14} to 1×10^{16} vg per patient [8]. However, as of 2024 the yields of mammalian-based AAV range from 1×10^{13} to 4×10^{15} vg/L depending on the expression cassette, vector length, and capsid serotype [8,54,55]. Assuming an average yield of 3×10^{14} vg/L, and a 25% recovery rate after purification [54], a 200 L bioreactor yields

FIGURE 2

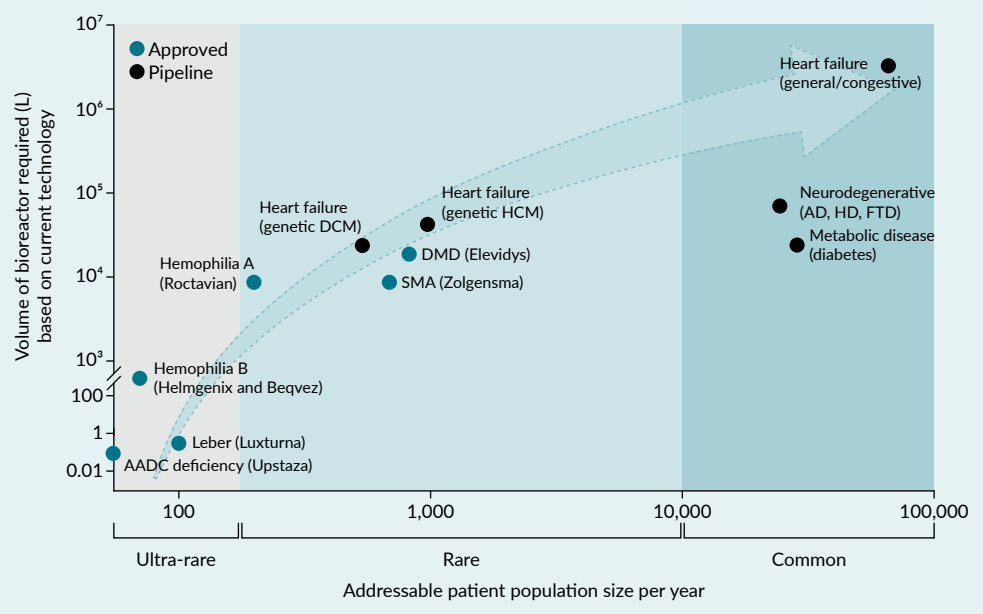
General overview of AAV production using plasmid transfection in mammalian cells.



Upstream activities include cell-line selection, media formulation, plasmid sequence and ratio optimization, transfection reagent selection and optimization, cell expansion and scale-up, and bioreactor process optimization and monitoring. Downstream activities include concentration of raw materials, cell lysis, nuclease treatment, purification, polishing, formulation, fill, and finish.

FIGURE 3

AAV therapies moving toward common indications and bioreactor capacity to address pipeline therapies.



Volume of bioreactor needed to treat patient populations per year for either approved or pipeline AAV gene therapy programs per indication. The bioreactor capacity was calculated based on the current industry standard (average yield of 3×10^{14} vg/L and a 25% recovery). The estimated yearly number of patients included those in the USA, Canada, EU5, and Australia. The vector needed for systemic dosing was calculated based on the average weight of 75 kg for adult patients, 18 kg for 5-year-old patients receiving Elevidys, and 12 kg for 2-year-old patients receiving Zolgensma. The calculations also assume a market penetration or drug adoption of 25% for genetic heart failure (dilated cardiomyopathy [DCM] and hypertrophic cardiomyopathy [HCM]), 1% for general heart failure, 1% for metabolic diseases (diabetes), and 1% for neurodegenerative diseases (Alzheimer's disease [AD], Huntington's disease [HD], and frontotemporal dementia [FTD] combined).

enough material to treat 1 to 10 patients with a systemically delivered mid-to-high dose of AAV gene therapy.

OPTIMIZATION CHALLENGES WITH AAV PRODUCTION

As of 2024, most AAV manufacturers have maximized AAV yields by primarily focusing on DoE studies in upstream processes (e.g., plasmid ratio refinement, transfection reagent optimization, and media feed strategies) [54,56,57]. Although DoE studies are needed to optimize an existing manufacturing platform, their effectiveness is constrained within the boundaries of current manufacturing paradigms. For example, mammalian cells have not evolved to function as AAV production factories. HEK293 cells were historically chosen for bioproduction due to their ease of culture in both adherent and suspension cultures, rapid growth, and high transfection efficiency [20,58]. As a result, current cell-based systems may not support 'super physiologic' production of rAAVs. Therefore, DoE studies alone may not be enough to markedly enhance AAV production.

Another challenge in rAAV production is partly driven by our limited biological understanding of cellular processes involved in rAAV replication and assembly [40,59]. For example, rAAV production requires timely expression of AAV proteins, helper virus, and cellular gene products at appropriate levels; precise assembly of capsid proteins; as well as replication of vector genomes and their packaging into preformed empty capsids [4]. One study estimates that approximately 75% of

rAAV capsids are empty or partially formed [54]. Another study suggests that only a fraction (approximately 7%) of cells produce assembled rAAV capsids when using a triple-transfection method, despite a 60% transfection efficiency [60]. These inefficiencies lead to low-volume yields of rAAV production that are approximately 1000–4000-fold lower than that of monoclonal antibody production [61].

AN INTEGRATED APPROACH TO ADVANCING AAV PRODUCTION

We believe that innovative strategies in engineering cells and processes can disrupt upstream processes and complement conventional DoE studies. We also believe that these strategies can improve both the yield and quality of the starting material at a log scale to reduce the strain on downstream processing. Although downstream processes are crucial for the final AAV product, we propose that directing more research and resources to disruptive solutions upstream will lead to a higher titer and quality starting point that streamlines the overall AAV production process.

To this end, we developed an integrative strategy that uses a:

1. Systems biology approach powered by high-throughput screening and transcriptomics [62,63]; and

2. Rational design involving data-driven evolution of our proprietary split 2-plasmid platform (Figure 4) [64].

Specifically, we developed a 96-well platform to screen for AAV enhancers using Arrayed Targeted Libraries for AAV Screening (ATLAS) [65]. ATLAS can be used to induce gain-of-function, promote loss-of-function, and perturb pathways using microRNAs [66–69]. Although ATLAS can be used to test single perturbations, some perturbations may not 'nudge' the biological process or pathway enough to result in a detectable increase in AAV yield during the screening process. This result is particularly pronounced in complex biological systems that use multiple protein isoforms and multi-protein complexes. Thus, to better understand the mechanisms of AAV production and identify enhancers of AAV production, we propose to integrate complementary strategies: screening with transcriptomics and proteomics [16,62,63,70]. This synergistic approach accelerates the discovery of novel targets, enriches our hypothesis generation, and offers a robust framework for understanding the complex biology underlying AAV production (Figure 4).

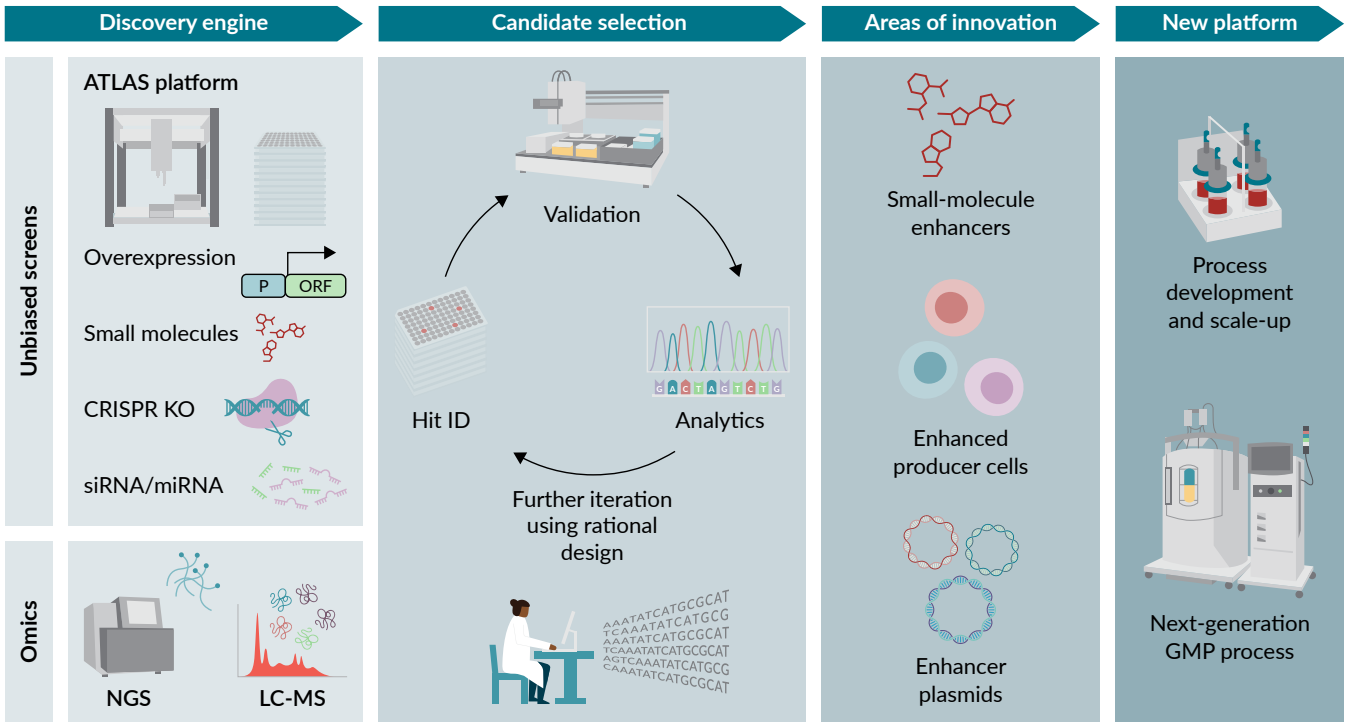
Our goal is to use this integrative approach to develop a rich dataset that captures both common and cell-specific enhancers of AAV production. Ultimately, this dataset can be leveraged along with rational design strategies and incorporated into the next generation of AAV manufacturing processes. These next generation processes could include improving media formulations (e.g., using small-molecule enhancers), introducing enhancer plasmids to optimize plasmid sequences (e.g., expressing shRNA and miRNA cassettes, enhancer open reading frames, or modified viral elements), and modifying genetic elements of the producer cell line (e.g., knocking out genes or overexpressing mammalian or viral elements).

CASE STUDY: HIGH-THROUGHPUT SCREENING AND TRANSCRIPTIONAL ANALYSIS IDENTIFY COMMON PATHWAYS PERTURBED DURING AAV PRODUCTION

To determine if data generated from a high-throughput AAV screen have commonalities with transcriptomics data collected during AAV production, we analyzed two internal datasets. We identified differentially expressed transcripts during AAV9 production with RNA-sequencing (Figure 5A) [70] and enhancers of AAV9 production with

FIGURE 4

New commercial platform developed using an integrated approach to identify AAV enhancers.

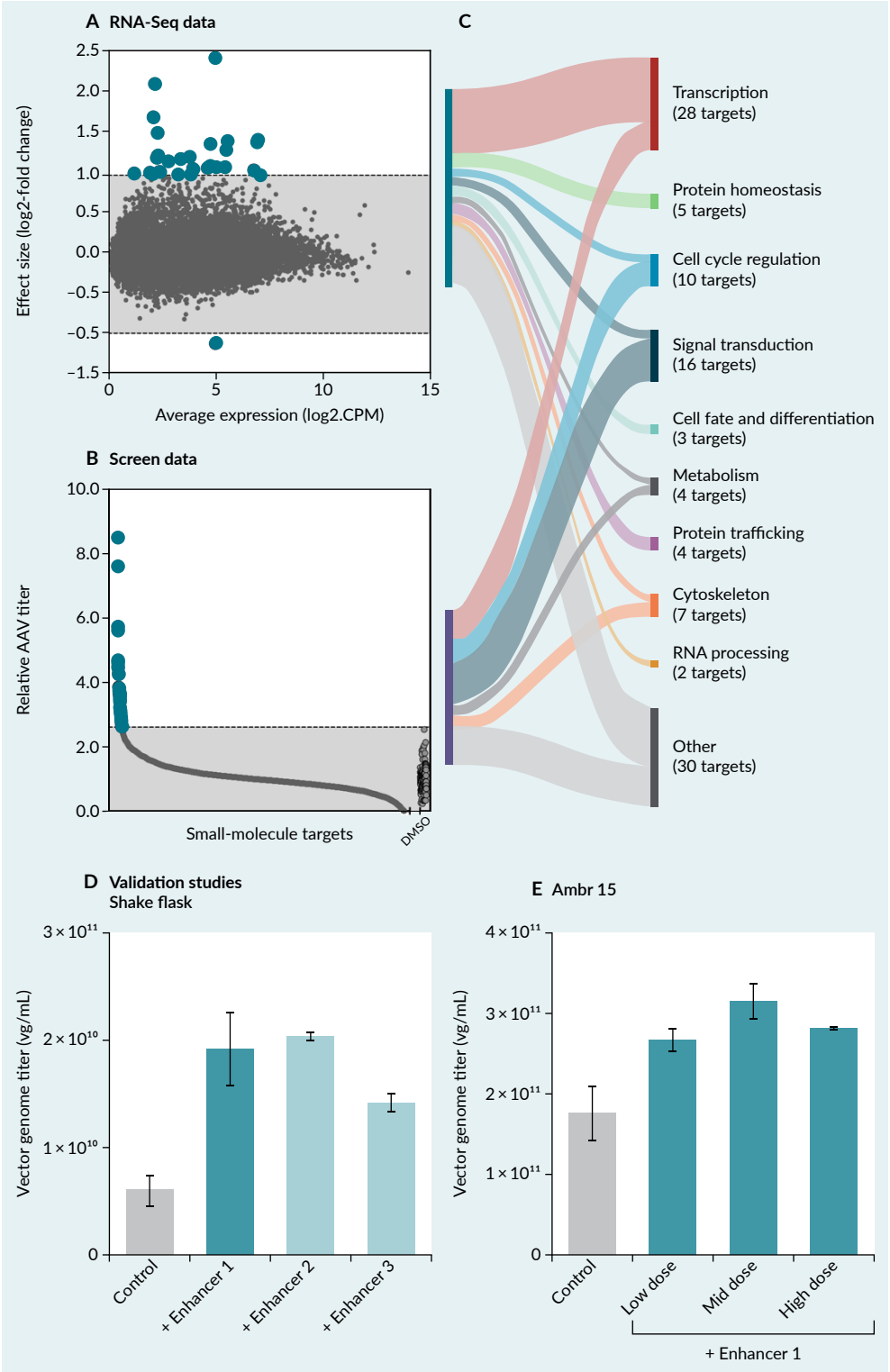


Arrayed Targeted Libraries for AAV Screening (ATLAS) is a high-throughput platform that can identify AAV enhancers in a 96-well format using gain-of-function or loss-of-function methods. Omic approaches can uncover mechanisms to identify key pathways during AAV production. The combination of these datasets is a rich source of proprietary data that can be mined and further refined with rational design to develop the next generation of AAV manufacturing. Areas of innovation could include discovering small-molecule enhancers or developing enhanced producer cell lines, plasmids, or sequences.
CRISPR: Clustered regularly interspaced short palindromic repeats; ID: Identification; KO: Knockout; LC-MS: Liquid chromatography mass spectrometry; NGS: Next-generation sequencing; ORF: Open reading frame.

a primary small-molecule screen using our ATLAS platform (Figure 5B) [68]. We then intersected the identified targets and categorized them into known biological pathways based on molecular function and gene ontology annotation. The top three shared pathways consisted of transcription, signal transduction, and cell-cycle regulation (Figure 5C). Some differentially altered pathways were identified with RNA-sequencing but not with the small-molecule screen (e.g., protein homeostasis, cell fate and differentiation, protein trafficking, RNA processing). This mismatch may be due to the composition of the small-molecule library and the limitation of the chemical probes targeting difficult drug targets and certain biological processes. Also, some pathways may be altered downstream because of AAV production, so modulation of those pathways would likely not affect AAV production. Next, we selected the top three most-promising small-molecule enhancers for studies in suspension shake flasks. All three enhancers yielded greater AAV9 yields compared to the control condition (Figure 5D). We validated the most-promising small-molecule enhancer in an Ambr15 bioreactor system at three doses (Figure 5E). Enhancer 1 shows a higher effect size on AAV9 yield in shake flakes (3.1-fold) compared to Amb15 (1.8-fold). The difference in relative fold enhancement may be attributed to a lower AAV production capacity observed in shake flasks compared to Amb15 bioreactors. This case study shows the feasibility of intersecting transcriptomics with high-throughput screening data to identify novel enhancers of AAV production and

FIGURE 5

High-throughput screening and transcriptional analysis to identify putative pathways that enhance AAV production.



(A) RNA-sequencing revealed differentially expressed transcripts during AAV9 production. **(B)** A high-throughput compound screen revealed small-molecule enhancers of AAV9 production. **(C)** Genes and pathways from two data sources were categorized into known biological pathways based on molecular function and gene ontology annotation. **(D)** The top three most-promising small-molecule enhancers improved AAV9 yields by 3.1-fold in suspension shake flasks. **(E)** Enhancer 1 was further advanced and improved AAV9 yields approximately 1.8-fold in an Ambr15 bioreactor system.

scale-up discoveries to develop more efficient processes.

SEQUENTIAL AAV PLATFORM DEVELOPMENT THROUGH A COMMERCIAL AND REGULATORY LENS

In the case study, we showed an example of how early-stage research and development can lead to discoveries that enhance existing platforms for AAV manufacturing. However, to commercialize a new AAV manufacturing platform, the platform needs to meet both regulatory and commercial requirements. For example, new discoveries must be paired with a strong analytical toolkit to drive advances in AAV bioprocessing. We are systematically stacking discoveries—especially those with a combinatorial effect and target-independent biological pathways—and combining them with further upstream and downstream process innovation. With this approach, we aim to improve AAV yield and quality in a stepwise manner over the next few years (Figure 6).

Importantly, the journey of innovation in bioprocessing must be navigated with caution to ensure that developments align with regulatory agencies and gene therapy developers. Without this alignment, regulatory agencies and gene therapy developers will be overwhelmed with rapid, groundbreaking process changes that could pose challenges. To avoid these challenges, developers need to adopt a balanced and gradual approach to introducing these new technologies. They also need to harmonize throughput and scalability by considering the intricacies of technology transfer throughout the scaling process, from microplates to shake flasks and, eventually, to small- and large-scale bioreactors. This harmony requires rigorous development, validation, and transfer of new technologies to ensure that each discovery is appropriately scaled and integrated into the manufacturing process. Through this balanced and systematic approach, we aim not only to innovate, but also to ensure the practical applicability and regulatory compliance of advances in producing AAV gene therapies. Our focus is to ensure continually improved product quality with the release and scale-up of each new-generation platform using our very broad analytic toolbox. With this approach, we aim to enable companies to move to larger scales or next-generation platforms within a product's life cycle as the demand for vectors increases, without incurring significant chemistry, manufacturing, and controls risk in terms of lack of comparability.

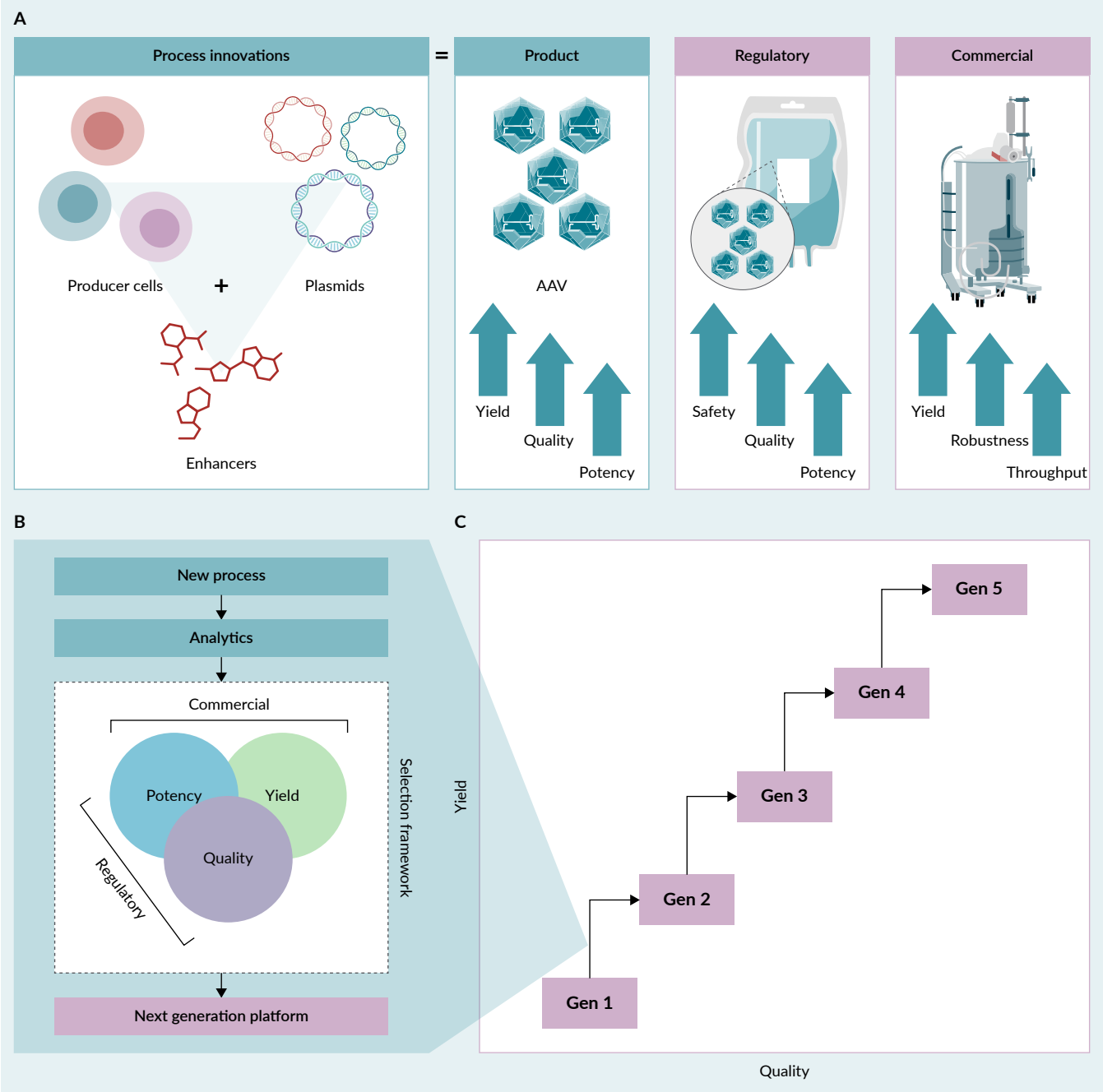
DISCUSSION AND FUTURE DIRECTIONS

Manufacturing AAV-based therapies is complex and costly, which affect both regulatory (safety defined by vector quality and potency) and commercial (cost of goods and the number of deliverable doses per year, defined by vector yield and potency) aspects. The operational demands and high costs are a result of challenges with scalability, process robustness, and productivity maintenance that result in gene therapies often reaching millions of dollars per treatment.

The high cost of treatment has led to challenges in the commercialization of some AAV gene therapies, as proven by the cases of Glybera and, most recently, Roctavian. Glybera, the first gene therapy approved in Europe, was withdrawn in 2017 due to its high cost and lack of national reimbursement, even though it showed potential benefits for patients with lipoprotein lipase deficiency [12]. Roctavian was recently approved for hemophilia A but has faced slow uptake due to issues with reimbursement and market access, resulting in low numbers of treated patients and modest sales [71]. These cases highlight the acute pricing challenges with AAV gene therapies. The high upfront costs, combined with the need for long-term efficacy data and innovative payment models, create a complex

FIGURE 6

Process innovation and sequential AAV platform development.



(A) Process innovations aim to enhance the yield, quality, and potency of AAV product to satisfy both regulatory and commercial standards. **(B)** New processes must be vetted using well-defined analytical methods to ensure a new manufacturing platform meets both commercial and regulatory requirements. **(C)** Advances in both yield and quality can be achieved by sequentially stacking discoveries and combining process innovations.

commercial landscape. These experiences underscore the importance of establishing robust value propositions and ensuring broad access to make AAV gene therapies commercially viable.

The commercialization issues can be exacerbated by the rapid growth of bespoke and nascent AAV manufacturing processes in early-stage biotech companies. Although the transition from a bespoke AAV process to

a clinical and, ultimately, commercial process has significant challenges, the transition offers substantial opportunities for innovation. For example, innovations in the manufacturing platform can enhance not only the yield and potency of the final drug product, but also the reproducibility, reducing batch failures, and ultimately shortening the development timeline and costs for sponsors. To address these issues, the FDA has encouraged early-stage companies to move their manufacturing processes to contract development and manufacturing organizations [43,46]. This shift aims to standardize procedures by drawing on strategies successfully implemented in the monoclonal antibody sector in the 1990s [72,73].

Despite similarities to monoclonal antibody production, AAV manufacturing poses unique challenges. For example, functional assembly of full AAV particles requires precise assembly of both capsid proteins and incorporation of nucleic acids [40,44,74]. Also, viral factors, including adenoviral helper elements and replicase proteins that are essential for replication and packaging, can lead to a DNA damage response and cell cycle arrest in producer cells [16]. Thus, to optimize AAV production, the expression and timing of these factors must be carefully calibrated.

To enhance manufacturing workflows, both processes and analytics must be standardized in collaboration with the FDA. As these advances are incorporated into manufacturing workflows, the cost of viral manufacturing will decline, and we may see a paradigm shift in the gene therapy business model. We also expect that the development of AAV gene therapies could expand beyond rare indications to also provide treatment options for common indications [46].

Looking ahead, we see the gene therapy industry moving toward progressively enhancing viral manufacturing platforms with several strategies. In this commentary, we described two strategies: high-throughput screening [75] and systems biology methods [16,63] to identify molecular targets that improve vector yield and quality. Some additional approaches that we did not discuss include optimizing vector sequences using machine learning [76,77], introducing synthetic DNA [78] to enhance vector potency, reducing plasmid-derived impurities, improving the safety and therapeutic index of AAV therapies, engineering viral proteins using mutagenesis screens [79], or using large language models [80] to enhance replication, packaging, and capsid assembly. Also, automating with robotics [73,81], using perfusion processes [15,82], and developing stable producer cell lines [19,26–31] can reduce the cost of goods and labor while also minimizing variation and batch failure during production campaigns. Finally, cell-free manufacturing may be among the next wave of innovations that could revolutionize viral manufacturing [83].

In addition to improving manufacturing methods and analytics, the safety and cost–effectiveness of AAV gene therapies must also be ensured by continuously optimizing product efficacy. This optimization is possible with both capsid selection and capsid engineering to improve the transduction of target cells, enhance the specificity of gene transfer, evade existing immune responses, and reduce *de novo* immune responses. The therapeutic expression cassette can also be optimized to improve safety and efficacy of the vector. This can be achieved through codon optimization and use of mRNA stabilizing elements to ensure optimal long-term expression. Additionally, using a tissue-specific promoter and miRNA de-targeting sequences can improve safety by restricting expression to the desired tissues.

Advanced drug developers also have the responsibility of maximizing the therapeutic index of potentially curative AAV medicines. This responsibility is crucial for providing safe and effective vectors to patients in urgent need. To achieve this goal, developers must learn from past experiences. For example, they need to address safety concerns that

resulted in clinical holds due to poor vector quality [84] or that led to avoidable deaths due to immunotoxicities and liver dysfunction in patients treated with high doses of AAV gene therapies [11,85]. To provide safe and effective treatments, developers must meticulously conduct research and development to ensure that they learn from both past and present experiences.

CONCLUSION

In this commentary we describe current AAV manufacturing practices, and the next steps required to develop more efficient, scalable, and cost-effective manufacturing processes for AAV gene therapies. To develop the next-generation manufacturing platform, we use an integrated approach that combines high-throughput screening and RNA-sequencing. Our discovery approach provides a rich source of proprietary data that can be mined to identify candidate targets that enhance the quality and yield of AAV. After selecting a candidate, and using rational design for further improvements, our new manufacturing platform could incorporate enhanced small-molecules, producer cell lines, and plasmids or sequences.

By integrating novel technologic approaches with traditional manufacturing paradigms, we have outlined a promising path to greatly enhance the yield and quality of AAV vectors. This systems biology approach, complemented by the ATLAS platform for screening and transcriptomic analysis, accelerates the identification of crucial production enhancers and reveals the underlying biological mechanisms for efficient AAV production. As the demand for AAV therapies continues to grow, particularly for treating common diseases, the need for improved production processes becomes even more important. The continued innovation in bioprocessing, coupled with regulatory and commercial alignment, will ensure that these advanced manufacturing techniques meet clinical demands, comply with regulatory requirements, and remain economically viable. In the future, AAV manufacturing will likely incorporate diverse technologies (from machine learning to synthetic biology to automation), promising a new era of accessibility and effectiveness in gene therapy. This strategic shift is essential to surpass the current limitations and fulfill the therapeutic promise of AAV therapies for addressing a broad spectrum of diseases.

REFERENCES

1. Samulski RJ, Muzyczka N. AAV-mediated gene therapy for research and therapeutic purposes. *Annu. Rev. Virol.* 2014; 1(1), 427–451.

2. Pupo A, Fernández A, Low SH, François A, Suárez-Amarán L, Samulski RJ. AAV vectors: the Rubik's cube of human gene therapy. *Mol. Ther.* 2022; 30(12), 3515–3541.

3. Li C, Samulski RJ. Engineering adeno-associated virus vectors for gene therapy. *Nat. Rev. Genet.* 2020; 21(4), 255–272.

4. Wang JH, Gessler DJ, Zhan W, Gallagher TL, Gao G. Adeno-associated virus as a delivery vector for gene therapy of human diseases. *Signal. Transduct. Target Ther.* 2024; 9(1), 78.

5. Kuzmin DA, Shutova MV, Johnston NR, *et al.* The clinical landscape for AAV gene therapies. *Nat. Rev. Drug. Discov.* 2021; 20(3), 173–174.

6. Prasad R, Lugo T, Corwin S, Hanbury-Brown L, Boyle J. *Under the Microscope State of the AAV Gene Therapy Landscape After Recent FDA AdCom on Safety.* 2021 William Blair Equity Research.

7. Bulcha JT, Wang Y, Ma H, Tai PWL, Gao G. Viral vector platforms within the gene therapy

landscape. *Signal. Transduct. Target Ther.* 2021; 6(1), 53.

8. Au HKE, Isalan M, Mielcarek M. Gene therapy advances: a meta-analysis of AAV usage in clinical settings. *Front. Med (Lausanne)* 2022; 8, 809118.

9. Wang D, Tai PWL, Gao G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nat. Rev. Drug Discov.* 2019; 18(5), 358–378.

10. Burdett T, Nuseibeh S. Changing trends in the development of AAV-based gene therapies: a meta-analysis of past and present therapies. *Gene Ther.* 2023; 30(3–4), 323–335.

11. High-dose AAV gene therapy deaths. *Nat. Biotechnol.* 2020; 38(8), 910.

12. Senior M. After Glybera's withdrawal, what's next for gene therapy? *Nat Biotechnol.* 2017; 35(6), 491–492.

13. FDA. https://www.fda.gov.

14. Gottlieb S. Statement from FDA Commissioner Scott Gottlieb, MD and Peter Marks, MD, PhD, Director of the Center for Biologics Evaluation and Research on new policies to advance development of safe and effective cell and gene therapies. *FDA* Jan 5, 2020. https://www.fda.gov/news-events/press-announcements/statement-fda-commissioner-scott-gottlieb-md-and-peter-marks-md-phd-director-center-biologics.

15. Grieger JC, Soltys SM, Samulski RJ. Production of recombinant adeno-associated virus vectors using suspension HEK293 cells and continuous harvest of vector from the culture media for GMP FIX and FLT1 clinical vector. *Mol. Ther.* 2016; 24(2), 287–297.

16. Chung CH, Murphy CM, Wingate VP, *et al.* Production of rAAV by plasmid transfection induces antiviral and inflammatory responses in suspension HEK293 cells. *Mol. Ther. Methods Clin. Dev.* 2023; 28, 272–283.

17. Nagy A, Chakrabarti L, Kurasawa J, *et al.* Engineered CHO cells as a novel AAV production platform for gene therapy delivery. *Sci. Rep.* 2023; 13(1), 19210.

18. Chadeuf G, Favre D, Tessier J, *et al.* Efficient recombinant adeno-associated virus production by a stable rep-cap HeLa cell line correlates with adenovirus-induced amplification of the integrated rep-cap genome. *J. Gene Med.* 2000; 2(4), 260–268.

19. Su W, Seymour LW, Cawood R. AAV production in stable packaging cells requires expression of adenovirus 22/33K protein to allow episomal amplification of integrated rep/cap genes. *Sci. Rep.* 2023; 13(1), 21670.

20. Tan E, Chin CSH, Lim ZFS, Ng SK. HEK293 cell line as a platform to produce recombinant proteins and viral vectors. *Front. Bioeng. Biotechnol.* 2021; 9, 796991.

21. Su W, Patrício MI, Duffy MR, Krakowiak JM, Seymour LW, Cawood R. Self-attenuating adenovirus enables production of recombinant adeno-associated virus for high manufacturing yield without contamination. *Nat. Commun.* 2022; 13(1), 1182.

22. Thomas DL, Wang L, Niamke J, *et al.* Scalable recombinant adeno-associated virus production using recombinant herpes simplex virus type 1 coinfection of suspension-adapted mammalian cells. *Hum. Gene Ther.* 2009; 20(8), 861–870.

23. Aslanidi G, Lamb K, Zolotukhin S. An inducible system for highly efficient production of recombinant adeno-associated virus (rAAV) vectors in insect Sf9 cells. *Proc. Natl. Acad. Sci. USA* 2009; 106(13), 5059–5064.

24. Marwidi Y, Nguyen HB, Santos D, *et al.* A robust and flexible baculovirus-insect cell system for AAV vector production with improved yield, capsid ratios and potency. *Mol. Ther. Methods Clin. Dev.* 2024; 32(2), 101228.

25. Mathur PD, Xie Q, Wong K, *et al.* Production of different AAV serotypes using a novel plant-based viral vector manufacturing platform (ASGCT abstract 25). *Mol. Ther.* 2024; 32, 4S1.

26. Merten OW. Development of stable packaging and producer cell lines for the production of AAV vectors. *Microorganisms* 2024; 12(2), 384.

27. Lee Z, Lu M, Irfanullah E, Soukup M, Hu WS. Construction of an rAAV producer cell line through synthetic biology. *ACS Synth. Biol.* 2022; 11(10), 3285–3295.

28. Jalšić L, Lytvyn V, Elahi SM, *et al.* Inducible HEK293 AAV packaging cell lines expressing Rep proteins. *Mol. Ther. Methods Clin. Dev.* 2023; 30, 259–275.

29. Han J, Tam K, Tam C, Hollis RP, Kohn DB. Improved lentiviral vector titers from a multigene knockout packaging line. *Mol. Ther. Oncolytics.* 2021; 23, 582–592.

30. Nitschke P. ELEVECTA®—the next level in gene therapy AAV production. *BioPharm Intl.* 2020.

31. Challener C. Proprietary cell-line development for high-titer AAV manufacturing. *BioPharm Intl.* 2024; 37, 10–15.

32. Liu S, Li J, Peraramelli S, *et al.* Systematic comparison of rAAV vectors manufactured using large-scale suspension cultures of Sf9 and HEK293 cells. *Mol. Ther.* 2024; 32(1), 74–83.

33. Urabe M, Ding C, Kotin RM. Insect cells as a factory to produce adeno-associated virus type 2 vectors. *Hum. Gene. Ther.* 2002; 13(16), 1935–1943.

34. Rumachik NG, Malaker SA, Poweleit N, *et al.* Methods matter: standard production platforms for recombinant AAV produce chemically and functionally distinct vectors. *Mol. Ther. Methods Clin. Dev.* 2020; 18, 98–118.

35. Kondratov O, Marsic D, Crosson SM, *et al.* Direct head-to-head evaluation of recombinant adeno-associated viral vectors manufactured in human versus insect cells. *Mol. Ther.* 2017; 25(12), 2661–2675.

36. Rao R, Farraha M, Logan GJ, *et al.* Performance of cardiotropic rAAV vectors is dependent on production method. *Viruses* 2022; 14(8), 1623.

37. ASGCT. 2023 ASGCT Abstract Book. *Mol. Ther.* 2023; 31, 4S1.

38. ASGCT. 2024 ASGCT Abstract Book. *Mol. Ther.* 2024; 32, 4S1.

39. Smith J, Grieger J, Samulski RJ. Overcoming bottlenecks in AAV manufacturing for gene therapy. *Cell Gene Ther. Ins.* 2018; 4(8), 815–825.

40. Srivastava A, Mallela KMG, Deorkar N, Brophy G. Manufacturing challenges and rational formulation development for AAV viral vectors. *J. Pharm. Sci.* 2021; 110(7), 2609–2624.

41. Feuerstein A. At $2.1 million, newly approved Novartis gene therapy will be world's most expensive drug. *STAT* 2019. https://www.statnews.com/2019/05/24/hold-novartis-zolgensma-approval

42. Naddaf M. Researchers welcome $3.5-million haemophilia gene therapy—but questions remain. *Nature* 2022; 612(7940), 388–389.

43. Marks P. Enhancing gene therapy regulatory interactions. *Expert Opin. Biol. Ther.* 2022; 22(9), 1073–1074.

44. Bennett A, Mietzsch M, Agbandje-McKenna M. Understanding capsid assembly and genome packaging for adeno-associated viruses. *Future Virol.* 2017; 12(6), 283–297.

45. Clément N, Grieger JC. Manufacturing of recombinant adeno-associated viral vectors for clinical trials. *Mol. Ther. Methods Clin. Dev.* 2016; 3, 16002.

46. Marks P, Witten C. Toward a new framework for the development of individualized therapies. *Gene Ther.* 2021; 28(10–11), 615–617.

47. Jackson CB, Richard AS, Ojha A, *et al.* AAV vectors engineered to target insulin receptor greatly enhance intramuscular gene delivery. *Mol. Ther. Methods Clin. Dev.* 2020; 19, 496–506.

48. Phillips MI, Mohuczy-Dominiak D, Coffey M, *et al.* Prolonged reduction of high blood pressure with an *in vivo*, nonpathogenic, adeno-associated viral vector delivery of AT1-R mRNA antisense. *Hypertension* 1997; 29(1 Pt 2), 374–380.

49. Rosenberg JB, Kaplitt MG, De BP, *et al.* AAVrh.10-mediated APOE2 central nervous system gene therapy for APOE4-associated Alzheimer's disease. *Hum. Gene Ther. Clin. Dev.* 2018; 29(1), 24–47.

50. Yang Y, Seok MJ, Kim YE, *et al.* Adeno-associated virus (AAV) 9-mediated gene delivery of Nurr1 and Foxa2 ameliorates symptoms and pathologies of Alzheimer disease model mice by suppressing neuro-inflammation and glial pathology. *Psychiatry* 2023; 28(12), 5359–5374.

51. Wu I, Zeng A, Greer-Short A, *et al.* AAV9:PKP2 improves heart function and survival in a Pkp2-deficient mouse model of arrhythmogenic right ventricular cardiomyopathy. *Commun. Med. (Lond).* 2024; 4(1), 38.

52. Manso AM, Hashem SI, Nelson BC, *et al.* Systemic AAV9.LAMP2B injection reverses metabolic and physiologic multiorgan dysfunction in a murine model of Danon disease. *Sci. Transl. Med.* 2020; 12(535), eaax1744.

53. Tilemann L, Ishikawa K, Weber T, Hajjar RJ. Gene therapy for heart failure. *Circ. Res.* 2012; 110(5), 777–793.

54. Coplan L, Zhang Z, Ragone N, *et al.* High-yield recombinant adeno-associated viral vector production by multivariate optimization of bioprocess and transfection conditions. *Biotechnol. Prog.* 2024; 40(3), e3445.

55. Xue W, Fulco C, Sha S, *et al.* Adeno-associated virus perfusion enhanced expression: a commercially scalable, high titer, high quality producer cell line process. |*Mol. Ther. Methods Clin. Dev.* 2024; 32(2), 101266.

56. Zhao H, Lee KJ, Daris M, *et al.* Creation of a high-yield AAV vector production platform in suspension cells using a design-of-experiment approach. *Mol. Ther. Methods Clin. Dev.* 2020; 18, 312–320.

57. Kimura T, Ferran B, Tsukahara Y, *et al.* Production of adeno-associated virus vectors for *in vitro* and *in vivo* applications. *Sci. Rep.* 2019; 9(1), 13601.

58. Dumont J, Euwart D, Mei B, Estes S, Kshirsagar R. Human cell lines for biopharmaceutical manufacturing: history, status, and future perspectives. *Crit. Rev. Biotechnol.* 2016; 36(6), 1110–1122.

59. Nguyen TNT, Sha S, Hong MS, *et al.* Mechanistic model for production of recombinant adeno-associated virus via triple transfection of HEK293 cells. *Mol. Ther. Methods Clin. Dev.* 2021; 21, 642–655.

60. Dash S, Sharon DM, Mullick A, Kamen AA. Only a small fraction of cells produce assembled capsids during transfection-based manufacturing of adeno-associated virus vectors. *Biotechnol. Bioeng.* 2022; 119(6), 1685–1690.

61. Wright JF. AAV vector production: troublesome host innate responses in another setting. *Mol. Ther. Methods Clin. Dev.* 2023; 28, 412–413.

62. Samoudi M, Masson HO, Kuo CC, Robinson CM, Lewis NE. From omics to cellular mechanisms in mammalian cell factory development. *Curr. Opin. Chem. Eng.* 2021; 32, 100688.

63. Strasser L, Boi S, Guapo F, *et al*. Proteomic landscape of adeno-associated virus (AAV)-producing HEK293 cells. *Int. J. Mol. Sci.* 2021; 22(21), 11499.

64. Hörer M, Sonntag F, Kober R. Plasmid system. EP3722434B1. 2019.

65. Reid CA, Grafton F, Leveque-Eichhorn L, *et al.* ATLAS: A high-throughput gain and loss-of-function screening platform for optimizing AAV production (ASGCT abstract 1616). *Mol. Ther.* 2023; 31, 4S1.

66. Wang C, Sheth M, Ispaso F, *et al*. A genome-wide high-throughput gain-of-function screen identifies novel targets for improved AAV9 production (ASGCT abstract 1505) *Mol. Ther.* 2024; 32, 4S1.

67. Grafton F, Feitzinger R, Fisher K, Leveque-Eichhorn L, Reid CA, Mandegar MA. Targeted CRISPR/Cas9 screen identifies superior HEK293 cell lines for AAV9 production (ASGCT abstract 1334). *Mol. Ther.* 2023; 31, 4S1.

68. Grafton F, Feitzinger R, Fisher K, Leveque-Eichhorn L, Reid CA, Mandegar MA. A high-throughput small molecule screen identifies targets that increase AAV9 production in suspension HEK293 cells (ASGCT abstract 921). *Mol. Ther.* 2023; 31, 4S1.

69. Finkbeiner B, Burkhart M, Reichl S, *et al.* Whole genome high-throughput screen identified microRNAs enhancing rAAV production (ASGCT abstract 1058). *Mol. Ther.* 2024; 32–4S1

70. Tworig J, Grafton F, Hörer M, Reid CA, Mandegar MA. Transcriptomic analysis identifies differential expression patterns in cellular stress response, signal transduction, and extracellular matrix proteins during AAV production (ASGCT abstract 1063). *Mol. Ther.* 2024; 32, 4S1

71. Becker Z. With sales still stagnant, BioMarin adds divestiture to list of options for gene therapy. *Roctavian Fiercepharma* 2024.

72. Jiang Z, Dalby PA. Challenges in scaling up AAV-based gene therapy manufacturing. *Trends Biotechnol.* 2023; 41(10), 1268–1281.

73. Mirasol F. Gleaning scale-up lessons from mAbs for viral vectors. *Pharm. Tech.* 2023; 47, 20–21.

74. Onishi T, Nonaka M, Maruno T, *et al.* Enhancement of recombinant adeno-associated virus activity by improved stoichiometry and homogeneity of capsid protein assembly. *Mol. Ther. Methods Clin. Dev.* 2023; 31, 101142.

75. Barnes CR, Lee H, Ojala DS, Lewis KK, Limsirichai P, Schaffer DV. Genome-wide activation screens to increase adeno-associated virus production. *Mol. Ther. Nucleic Acids* 2021; 26, 94–103.

76. Nipko J. Developing machine learning powered solutions for cell and gene therapy candidate validation form. Bio White Paper 2023.

77. Ketz N. Leveraging model interpretability methods to predict gene therapy manufacturing failures form. Bio White Paper 2023.

78. Karbowniczek K, Extance J, Milsom S, *et al.* Doggybone™ DNA: an advanced platform for AAV production. *Cell Gene Ther. Ins.* 2017; 3(9), 731–738.

79. Jain NK, Ogden PJ, Church GM. Comprehensive mutagenesis maps the effect of all single-codon mutations in the AAV2 rep gene on AAV production. *Elife* 2024; 12:RP87730.

80. Madani A, Krause B, Greene ER, *et al.* Large language models generate functional protein sequences across diverse families. *Nat. Biotechnol.* 2023; 41(8), 1099–1106.

81. Tristan CA, Ormanoglu P, Slamecka J, *et al.* Robotic high-throughput biomanufacturing and functional differentiation of human pluripotent stem cells. *Stem Cell Reports* 2021; 16(12), 3076–3092.

82. Mendes JP, Fernandes B, Pineda E, *et al.* AAV process intensification by perfusion bioreaction and integrated clarification. *Front. Bioeng. Biotechnol.* 2022; 10, 1020174.

83. Vilkhovoy M, Adhikari A, Vadhin S, Varner JD. The evolution of cell free biomanufacturing. *Processes* 2020; 8, 675.

84. Solid Biosciences, Inc. Solid Biosciences announces FDA lifts clinical hold on IGNITE DMD clinical trial. *Solid Biosciences* 2020.

85. Duan D. Lethal immunotoxicity in high-dose systemic AAV therapy. *Mol. Ther.* 2023; 31(11), 3123–3126.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Maximizing process control and efficiency in CAR-T cell manufacturing

Mina Ahmadi, Jason Isaacson, Russell Jarres, and Evan Zynda

The complexity of CAR-T cell manufacturing poses a barrier to the wider accessibility and uptake of transformative CAR-T cell-based therapies. The ability to complete T cell isolation and activation in a single, automated step can enable a simpler workflow, resulting in time savings and process control improvements. In addition, the development of non-viral and scalable CAR-T workflows can support increased genome editing efficiency, enhanced precision, and higher expansion of CAR-T cells.

Here, we introduce available off-the-shelf technology to enable one-step isolation and activation, and present data demonstrating the ability to integrate several CAR-T process steps combining multiple platforms in a closed, automated workflow. A non-viral system for the expansion of CAR-T cells is explored alongside a scalable platform to improve the growth of desirable T cell phenotypes.

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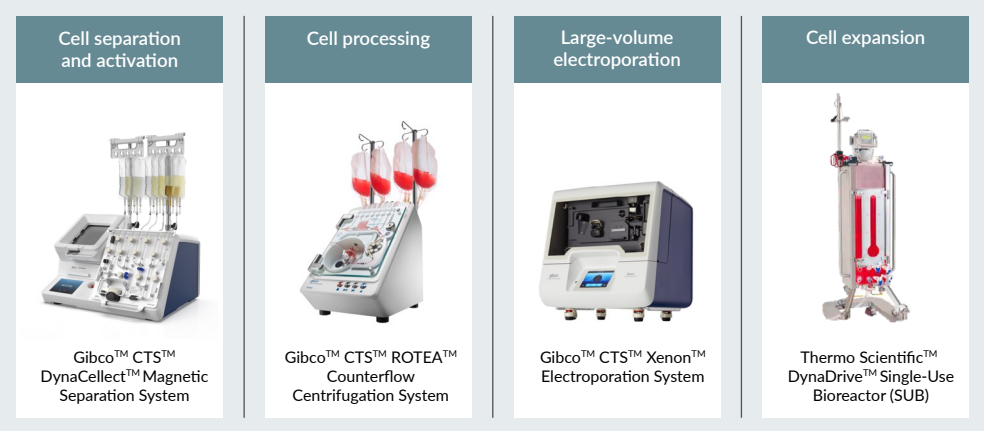
CAR-T cell therapy represents a transformative healthcare innovation for patients. However, the reality is that only around 3% of patients who could potentially benefit are currently receiving them [1–3]. This is due to a variety of factors, but many of them are underpinned by the complexity of the CAR-T cell manufacturing process.

The typical CAR-T manufacturing process can be broken into five components:

- Patient material collection;
- Isolation and activation;
- Payload delivery;
- Cell expansion of cells;
- Cryopreservation of the final drug product.

FIGURE 1

Modular, closed, scalable automated instruments for cell therapy processing.



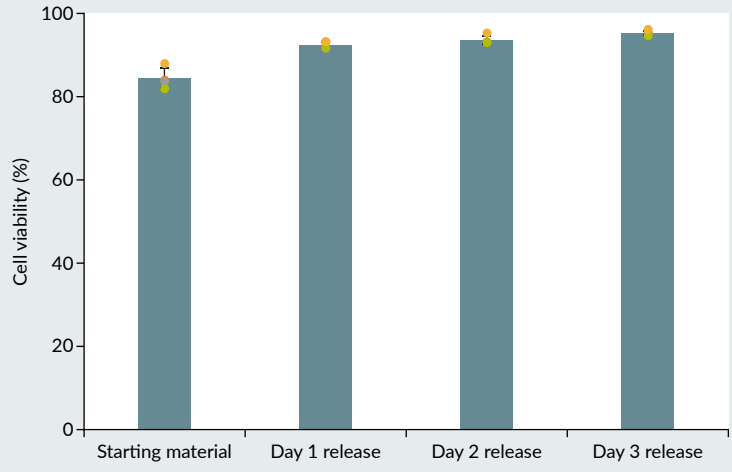
In terms of the typical upstream process steps and timeframe, T cells are isolated and activated on day 0, followed by gene modification on day 3, then expansion of the engineered cells through day 14 (although recent literature has suggested that a faster *ex vivo* culture process may yield improvements in the final drug product efficacy).

Thermo Fisher Scientific's vision for accelerated cell and gene therapy manufacturing and development timeframes is built on instruments that are standalone, closed, and automated unit operations. Nonetheless these instruments can also be connected physically and digitally to form end-to-end workflows. These instruments (Figure 1) use flexible process design software, which can be locked down for manufacturing later in development, as well as single-use, GMP-grade reagents, and consumables.

This article provides a detailed, step-by-step walkthrough of the CAR-T cell manufacturing process, exploring a comprehensive range of cell therapy instruments and innovations designed to cover the entire workflow. Crucial data points in the isolation, activation, gene modification, and expansion stages key to optimizing the

FIGURE 2

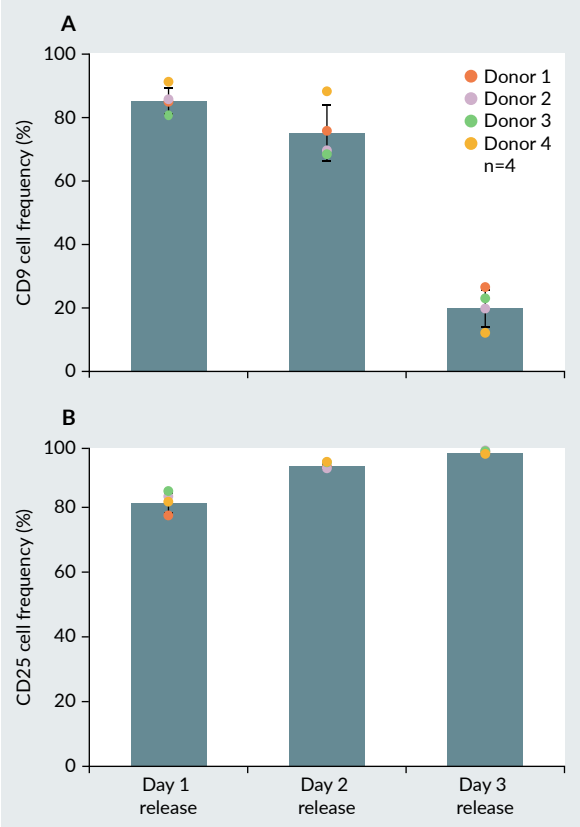
Optimal viability and isolation efficiency of CTS detachable Dynabeads CD3/CD28 beads.



Isolation efficiency (%)		
	$CD3^+/CD28^+$	$CD3^+$
Donor 1	93	84
Donor 2	96	83
Donor 3	93	71
Donor 4	88	78
Average	93	79
Standard deviation	3.3	5.7

FIGURE 3

Activation markers for CTS Detachable Dynabeads CD3/CD28 beads.



CAR-T cell therapy manufacturing process as a whole will be discussed. Furthermore, the utility of closed, automated cell processing in effectively reducing variability, cost, and process time while enhancing scalability will be examined.

SINGLE-STEP T CELL ISOLATION AND ACTIVATION

In order to fully integrate and automate workflows in the manufacturing of CAR-T cell therapies whilst simultaneously enhancing efficiency and flexibility, Thermo Fisher Scientific recently launched the next-generation platform of CTS™ Detachable Dynabeads™, which may be used in conjunction with both the Gibco™ CTS™ DynaCellect™ Magnetic Separation System and CTS™ Cellmation™ Software.

When used in combination with the CTS Detachable Dynabeads Release Buffer, the new CTS Detachable Dynabeads CD3/CD28 magnetic beads represent the first launch of the platform that offers process flexibility with an active release mechanism. Through its custom designed combination with the fully closed and automated DynaCellect Magnetic Separation System, the Detachable Dynabeads CD3/CD28 magnetic beads offer greater control for efficient T cell isolation and activation while providing the added flexibility of magnetic Dynabead removal. Furthermore, utilizing the provided Release Buffer at desired times allows for bespoke tuning of activation duration. Together, this allows for one-step isolation and activation, tunable activation to avoid exhausted phenotypes, and automated process control, which together result in time savings and process control improvements.

In a recent study, the new CTS Detachable Dynabeads CD3/CD28 magnetic beads were compared with performance of the passive-release CTS Dynabeads CD3/CD28 magnetic beads. Figure 2 demonstrates that isolated T cell viability of >90% was achieved when the Detachable Dynabeads were released on days one through three, which was higher than that for initial input material. Isolation metrics also average >90% and show specificity for desirable early memory T cell phenotypes.

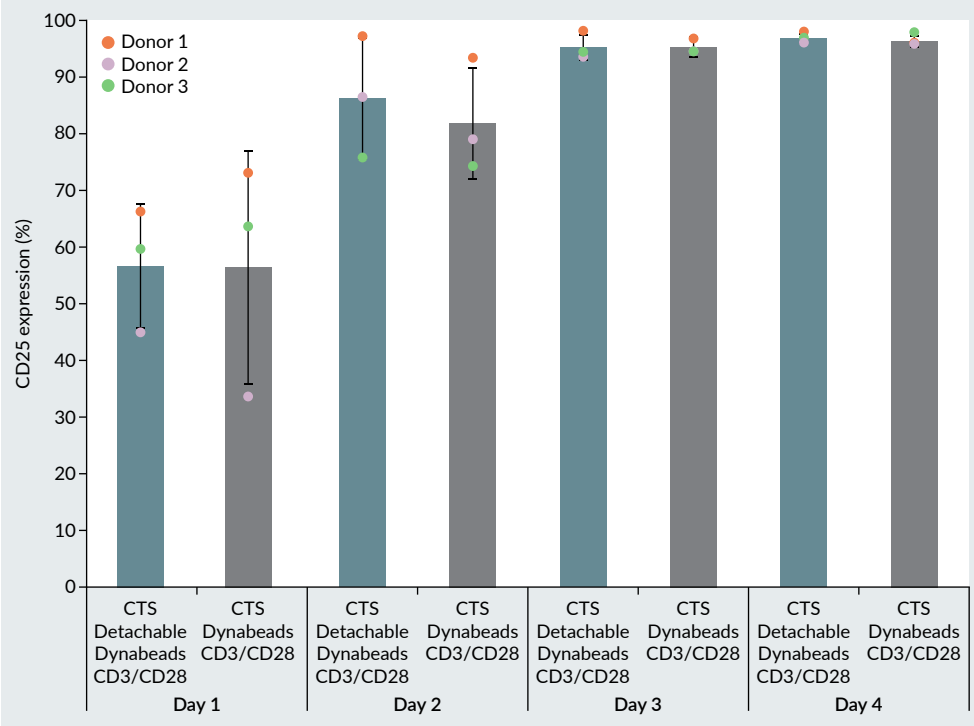
The combination of the CTS Detachable Dynabeads and CTS DynaCellect Magnetic Separation System can yield high T cell purity with a continuous production approach. Method improvements using this approach have demonstrated that no upstream wash of the input Leukopak material is needed, further reducing time and cost, whilst yielding post-isolation cultures of 98% pure T cells. CTS Detachable Dynabeads induce expected upregulation of T cell activation markers CD69 and CD25.

In Figure 3, early T activation markers show that CD69 expression is upregulated most at day 1, with a decline through day 3 active

bead release (Figure 3A), whereas in Figure 3B, CD25 is upregulated substantially on day 1 but then increases through day 3 active bead release. Both these key T cell activation

FIGURE 4

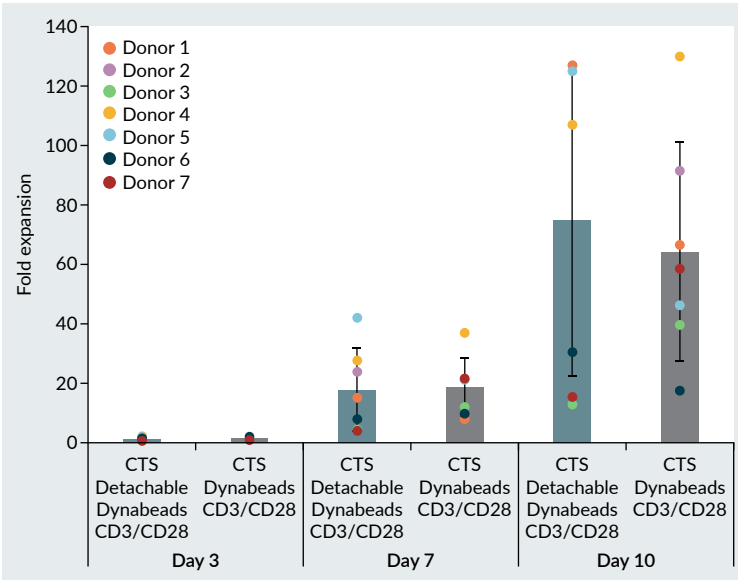
Comparison of activation and CD25 expression between active and passive release Dynabeads CD3/CD28 magnetic beads.



- Both the active- and passive-release Dynabeads magnetic beads had ~80% activation on day 2
- Both the active- and passive- release Dynabeads magnetic beads had ~95% activation on day 3
- CD25 expression for individual donor samples was similar between the active- and passive-release beads

FIGURE 5

Demonstrating comparable cell expansion on days 3, 7, and 10 across both Detachable Dynabeads and passive release Dynabeads magnetic beads.



- An average cell expansion of ~20-fold by day 7 was seen for both the active- and passive-release Dynabeads magnetic beads
- An average cell expansion of ≥60-fold by day 10 was seen for both the active- and passive-release Dynabeads magnetic beads
- Individual donor samples drove the standard deviation

markers adhere to expected kinetics on days 1 through 3.

Figure 4 shows data from a direct comparison of the abilities of the CTS Detachable Dynabeads and the 'passive release' CTS Dynabeads CD3/CD28 magnetic beads to activate T cells. Both the active and passive release Dynabeads had approximately 80% activation on day 2, and approximately 95% activation on day 3, while CD25 expression for individual donor samples was also similar between the two.

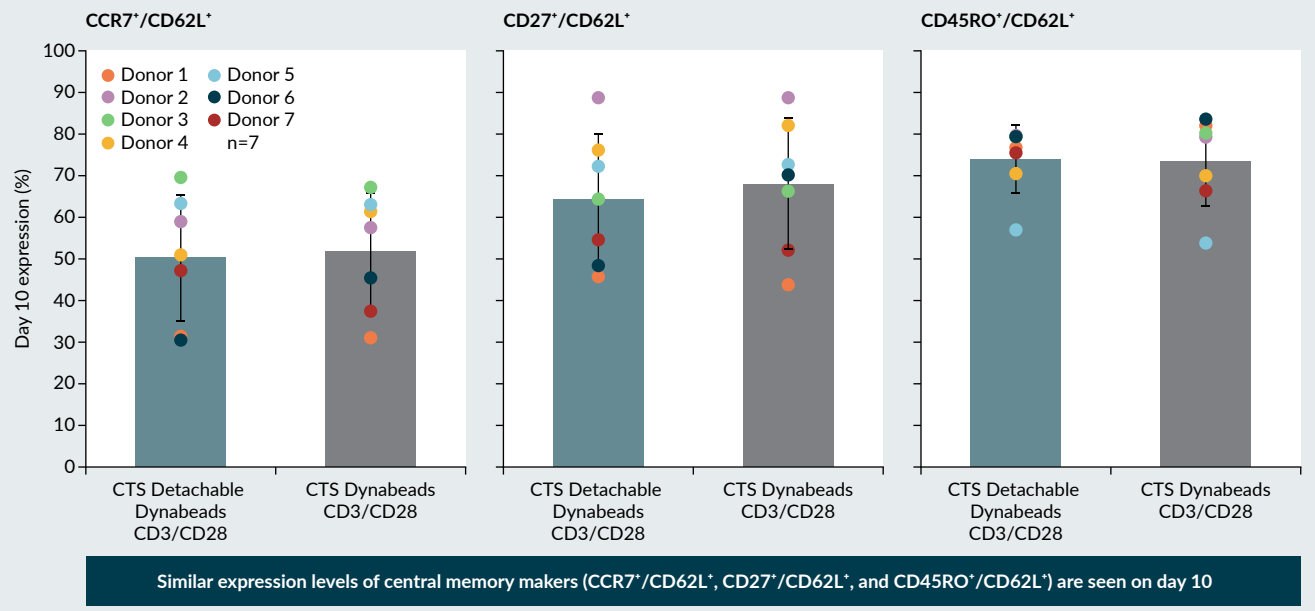
In Figure 5, data for T cell fold expansion is shown on days 3, 7, and 10 across seven healthy donors. This shows an average cell expansion of approximately 20-fold by day 7 for both the active and passive release Dynabeads CD3/CD28 magnetic beads. An average cell expansion of >60-fold by day 10 is also observed for both active and passive release beads. Individual donor samples drive the observed standard deviation across this dataset.

T cell memory phenotype plays an important role in the quality of a CAR-T cell therapy product. Figure 6 shows data from seven healthy donors, with each showing similar expression levels on day 10 of central memory markers CCR7/CD62L, CD27/CD62L, and CD45RO/CD62L for T cells activated either using the active or the passive release Dynabeads.

In summary, the performance of both the new CTS Detachable Dynabeads CD3/CD28 magnetic beads and the legacy passive release CTS Dynabeads CD3/CD28 magnetic beads is very similar in terms of activation, expansion, T cell ratios, and phenotype. The comparable performance of the Detachable Dynabeads CD3/CD28 magnetic beads together with the CTS Detachable Dynabeads Release Buffer and its active release functionality enhances the ability of scientists to address biological variability and provides flexibility in the CAR-T cell manufacturing process. Furthermore, when comparing the CD3/CD28 one-step method to a standard CD4 and CD8 isolation method, a significant time saving was observed on day 0 (2 h versus 4 h), with the added benefit that the Detachable Dynabeads allow the tuning of exposure to the activation signal.

FIGURE 6

Maintenance of strong memory T cell phenotype across both active and passive release Dynabeads CD3/CD28 magnetic beads.



This shorter run-time and the corresponding reduction in the quantity of reagents required leads to a potential reduction in overall processing time and costs.

Integrating CTS Cellmation™ Software—an off-the-shelf automation solution—into the workflow addresses key cell therapy development challenges related to standardization and automation, helping to overcome process challenges and accelerate development of CAR-T cell therapies. CTS Cellmation Software enables process control and data management for cell therapy workflows by allowing digital workflow integration, process and plant control, electronic data management, and cGMP compliance. It also enables a robust and reliable process by reducing the number of manual touchpoints.

CTS Cellmation Software connects several Thermo Fisher platforms, including the Gibco™ CTS™ DynaCellect system, the Gibco™ CTS™ Rotea™ Counterflow Centrifugation System, the Gibco™ CTS™ Xenon™ Electroporation System, the Thermo Scientific™ HeraCell VIOS™ CO_2 Incubator, and the Thermo Scientific™ CryoMed™ Controlled Rate Freezer.

In a recent study demonstrating the ability to combine multiple platforms in a closed, automated workflow, several CAR-T process steps were integrated: on day 0, T cell isolation and activation (utilizing the CTS Detachable Dynabeads CD3/CD28 magnetic beads and CTS DynaCellect system); on day 2, Dynabead removal and wash, followed by T cell engineering (utilizing the DynaCellect, Rotea, and Xenon systems respectively); and on day 9, CAR-T cell harvest (utilizing the Rotea system).

Notably, on day 2, all three instruments used were also physically connected by sterile welding PVC tubing, integrating three instruments into one functional module for debeading, GE buffer exchange and payload addition, and finally, electroporation. The resulting data set (Figure 7) supports the adoption of closed, automated, and modular integrations for CAR-T cell manufacturing. Highlights include high cell viability of day 0-isolated T cells of >90% and high purity of the isolated T cell culture. After two days of activation culture, the resulting T cells displayed expected activation status, as highlighted by CD69, CD25, and HLA-DR expression levels.

The day 2 activated culture was >95% pure T cells. By day 9, the CAR-T culture was of high T cell content and had shifted to predominantly CD8 T cells. While activation markers CD69 and CD25 were reduced from early culture levels, HLA-DR, a late activation marker, had increased to high levels. Residual CTS Dynabeads were assessed after harvest on the Rotea system and shown to be under the 100 beads per 3 million cells threshold required by the US FDA.

While some run or donor variability was observed, the data showed an average of 30% anti-CD19-CAR-expressing T cells that were fully edited, including TCR knockout. From this method, starting from a quarter Leukopak, it proved possible to generate an average of 1.6 billion total CAR-T cells with an average fold expansion of 11 post-electroporation.

The CAR-T cells generated were largely of an early memory phenotype at day 9 harvesting, suggesting that the approach being evaluated did not significantly alter the memory phenotype of the input material. Finally, LAG3, TIM3, PD1, and TIGIT marker expression patterns indicated that this approach did not significantly affect the exhaustion levels of the CAR-T cells created. While LAG3 and PD1 levels did increase at day 2 post-activation, those two markers returned to low levels by day 9. The TIGIT marker was higher overall—however, day 9 expression was not higher than on day 0.

ADVANCED NON-VIRAL CAR-T CELL ENGINEERING

Ensuring safety is a top priority in engineered cell therapy manufacturing. It is therefore crucial to implement strategies to avoid any potential negative consequences

of the manufacturing process. By developing advanced tools and technologies that support non-viral CAR-T workflows, Thermo Fisher Scientific is enabling increased genome editing efficiency, enhanced precision, and higher expansion of CAR-T cells.

A typical non-viral CAR-T cell manufacturing process begins with leukapheresis, followed by T cell isolation. The T cells are then activated for a few days before undergoing genome engineering. Post-genome modification, the cells are expanded and once the

FIGURE 7

Cell counts and activation marker expression (days 0–2); cell types, activation marker expression, and residual Dynabeads (day 9).

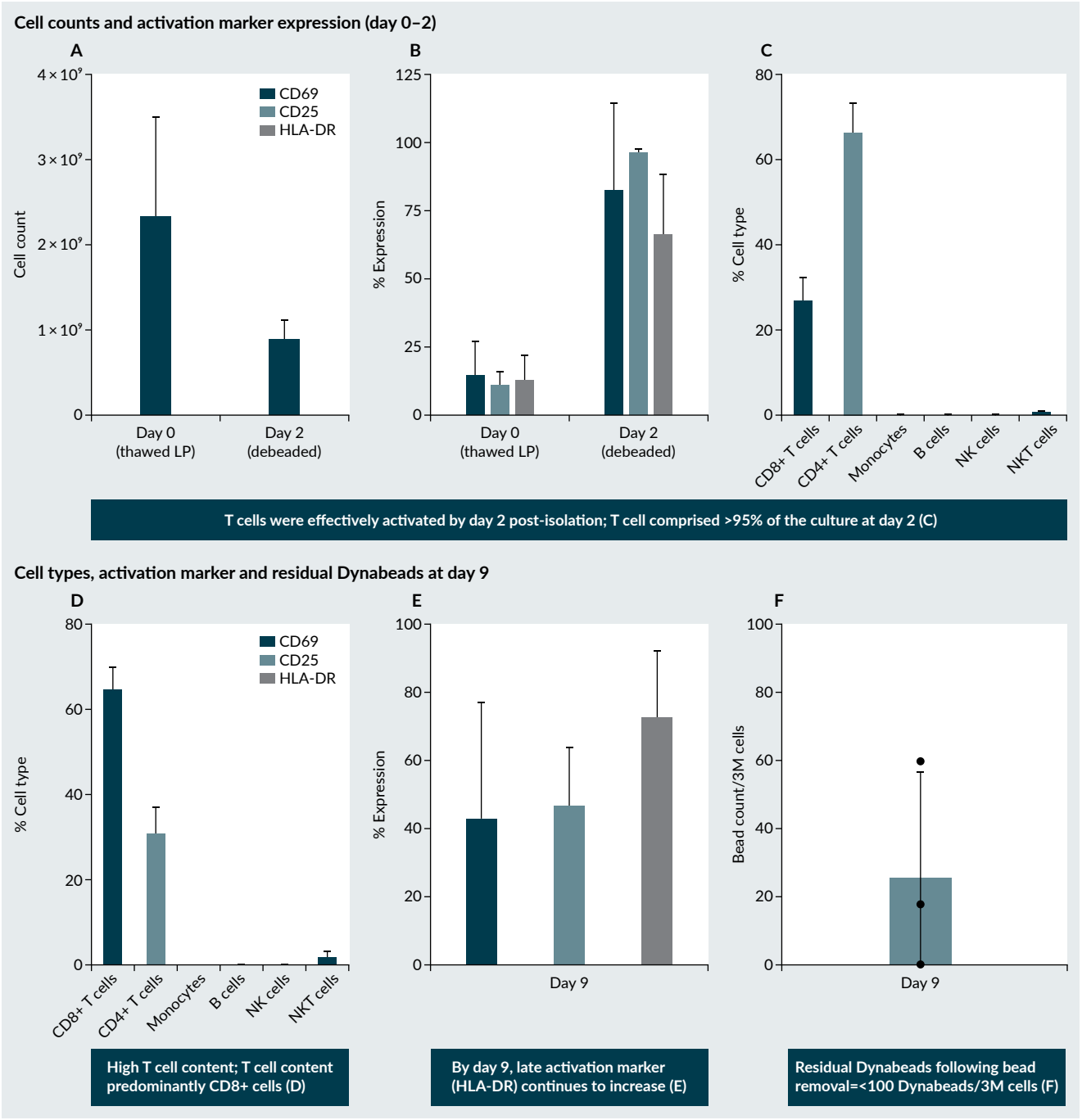
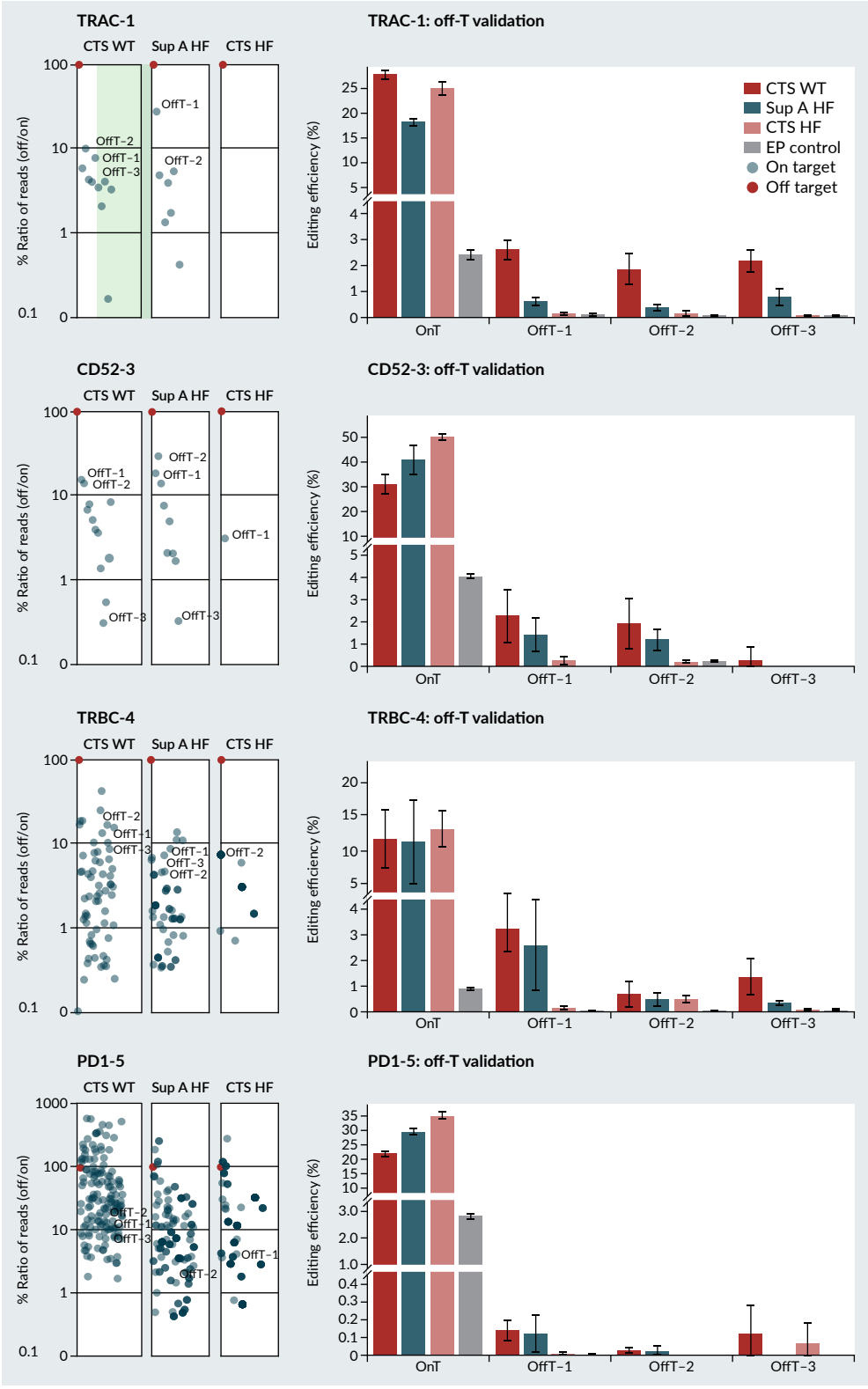


FIGURE 8

Comparison of wild-type CTS TrueCut Cas9, a GMP-compliant HiFi Cas9 from a competitor, and the recently launched Gibco™ CTS™ HiFi Cas9 Protein in terms of ability to reduce off-target effects in T cells.



required dose is achieved, the CAR-T cells are formulated and cryopreserved. Once the required product characterization testing is complete, they are ready to be infused into the patient or patients.

Genome engineering is one of the steps that may pose a risk to safety of the final therapeutic product. There are several ways in which to deliver gene editing machinery to cells. The traditional retroviral-based mechanisms offer the advantage of prolonged transgene expression—however, they can randomly integrate into the genome, which could result in harmful consequences and therefore raises safety concerns. The emergence of targeted genome editing approaches and new non-viral delivery methodologies is driving a shift away from retroviral-based methodologies. Leveraging CRISPR, TALENs, zinc-finger nucleases (ZFNs), and other similar tools allows for more targeted genome editing, thus minimizing the risk of subsequent adverse outcomes that could jeopardize patient safety. These gene editing components can be delivered to T cells through non-viral delivery approaches such as electroporation or via lipid nanoparticles (LNPs).

While genome editing tools alleviate concerns over random integration, they nonetheless present safety issues of their own in the form of off-target effects. In order to mitigate safety risk associated with Cas9 off-target effects, an improved variant of Cas9—High-Fidelity (HiFi) Cas9—has been introduced. Hi-Fi Cas9 was developed by selecting for fidelity-enhancing mutations, which improved the specificity of Cas9 for the intended target sequence to reduce the risk of off-target effects during the genome modification step. To validate the on-target/off-target profile and genome editing efficiency of HiFi Cas9, its performance was tested in primary T cells in small-scale genome editing experiments.

Figure 8 demonstrates HiFi Cas9 performance in terms of off-target effects. Targeted and guide RNA sequences with different known off-target profiles were selected to compare the off-target activity of the wild-type CTS TrueCut Cas9 (labelled 'CTS WT'), a GMP-compliant HiFi Cas9 from a competitor (labelled 'Supplier A HF'), and the recently launched Gibco™ CTS™ HiFi Cas9 Protein (labelled 'CTS HF'). Target-enriched GUIDE-sequencing (TEG-seq) was employed to identify off-target effects. TEG-seq is a useful qualitative tool for off-target discovery, providing information on how many different off-targets effects a certain target delivers, and how often they occur in comparison to the on-target effects. After TEG-seq was performed, Targeted Amplicon Validation sequencing (TAV-seq) was utilized to validate the TEG-seq and give better quantitative values.

TEG-seq involves adding DNA tags to the ends of double-stranded breaks induced by Cas9. These tagged ends can then be sequenced to identify off-target cleavage events that may have occurred elsewhere in the genome. The dot plot graphs show genome-wide off-target effects identified by TEG-seq. Three specific off-targets from each sgRNA were selected and off-target cleavage validation was performed using TAV-seq, in which a pair of primers were designed for each off-target to amplify the cleavage site, followed by NGS deep sequencing. The resulting cleavage (editing) % is plotted in the bar graphs beside each corresponding sgRNA TEG-seq result. All results demonstrate that the Gibco™ CTS™ HiFi Cas9 Protein (TFS HF) is the best option for reducing off-target activity, while the GMP-grade HiFi Cas9 of Supplier A was only mildly effective at reducing off-target effects in identical experiments.

Next, the CTS HiFi Cas9 Protein was validated and compared to the regular Gibco CTS TrueCut™ Cas9 Protein for on-target editing efficiency in primary T cells. Five gRNAs targeting four CAR-T cancer therapeutic-relevant genes (*TRAC*, *TRBC*, *CD52*, and *B2M*) were utilized for these experiments. The Cas9/gRNA ribonucleoprotein (RNP) complex was delivered

FIGURE 9

Comparing on-target activity for the CTS HiFi Cas9 protein and the Gibco CTS TrueCut Cas9 protein

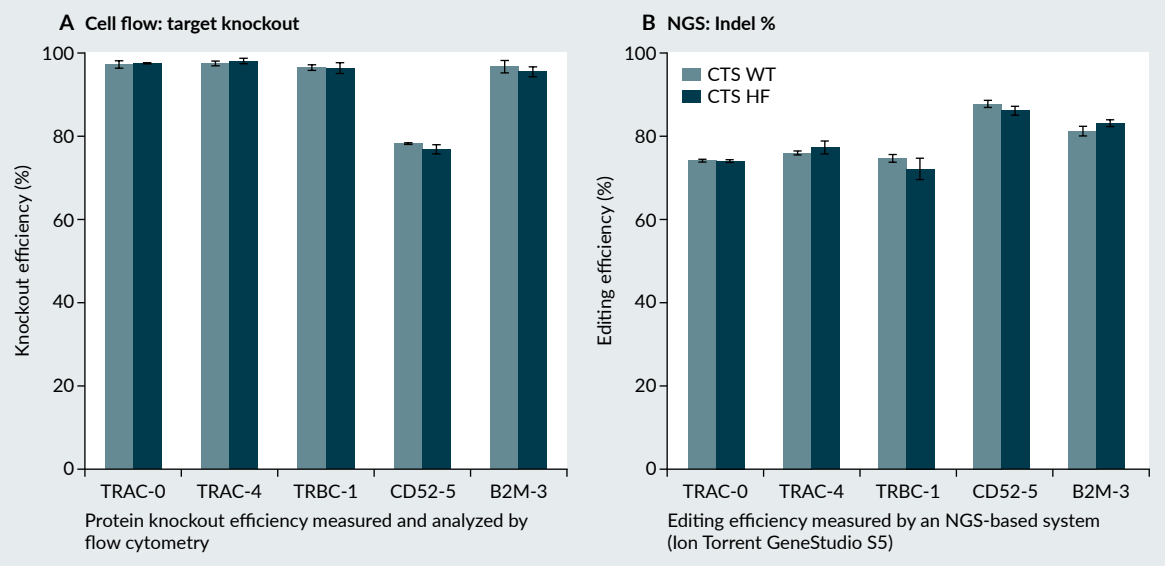
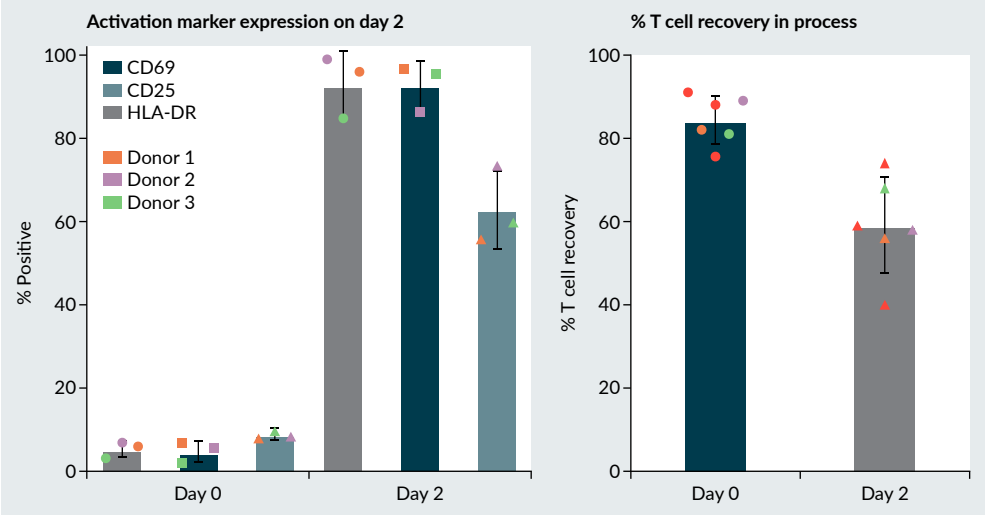


FIGURE 10

Activation marker and process recovery data.



through a Neon™ Electroporation System (10 µL) system with saturated amounts of Cas9 (9 pmol) and sgRNA (12 pmol) into 200,000 T cells. T cells were harvested and lysed on day 3 post-transcription. The lysed product was PCR amplified using a pair of primers to specifically amplify the product of 250 to 300 base pairs in length that was contained in those sequences. The amplicon samples were barcoded, pooled, and sequenced using the Ion GeneStudio S5 System.

The target protein knockout percentage shown in Figure 9A was measured by *TRAC*king the percentage of cell surface expression using Attune cell flow cytometry. The percentages shown in Figure 9B were calculated using an in-house-developed plug-in

analysis tool. All results confirmed comparable on-target activity between the Gibco CTS HiFi Cas9 and the CTS TrueCut Cas9 proteins.

The next experiment involved evaluating the HiFi Cas9 protein for its on-target knockout/knock-in efficiency in T cells. Single-stranded oligonucleotide (ssODN) was used as a knock-in donor at two targeted gene loci, *TRAC* and *CD52*. Genome editing efficiency was also compared using both R buffer and GE buffer. (GE buffer has been designed to improve performance with gene editing-specific payloads, especially for knock-in-based applications). In this set of experiments, an approximate 3-fold improvement in HDR efficiency for *TRAC-4* gRNA, and a 2.8-fold improvement for *CD52-5* gRNA were observed. Additionally, indel percentage was significantly reduced while overall editing efficiency was not significantly impacted. Cell viability remained at over 90% in all of the scenarios tested.

With high genome editing precision and efficiency having been achieved by HiFi Cas9 in these small-scale experiments, the next step was to incorporate HiFi Cas9 with other CTS genome editing tools in order to establish a full non-viral genome engineering workflow for CAR-T cells. On day 2 post-isolation, the freshly debeaded T cells were washed and concentrated into genome editing buffer using the CTS Rotea system. The CTS Xenon system was then employed to deliver RNP and anti-CD19 CAR (in the form of double-stranded DNA) to the cells). All three instruments were physically connected in this process. The cells were released from the CTS DynaCellect system and directly transferred to the CTS Rotea input bag. The payload containing RNP and anti-CD19 CAR was also directly transferred to the CTS Xenon system. This physical integration allows for a more automated, hands-off process.

Optimized electroporation protocols for activated T cells are available on the CTS Xenon system. Furthermore, the ability to adjust electroporation parameters on the Xenon system allows users to tailor existing protocols and/or to set up new parameters to further enhance process efficiency. Following electroporation, the T cells were seeded at 400,000–600,000 cells/cm^2 of G-Rex vessels. These cultures were treated as fed batch. The cells were fed on the second day and fourth day post-electroporation with CTS™ OpTmizer™ Pro Serum-Free Media (SFM) supplemented with 2.5% CTS ICSR and 100 U/ml GMP PeproTech IL2 cytokine.

In this non-viral genome engineering workflow experiment, the entire day 2 unit operation involving the three instruments described above was completed within 3 hours. The donor starting materials used contained varying compositions of peripheral blood mononuclear cells (PBMCs), but the day 2 culture was predominantly T cells, with approximately 98.8% T cell purity for all donors. Activation status was measured on day 2 prior to electroporation, and very good induction of early, mid, and late activation markers (CD69, CD25, and HLA-DR respectively) was observed, as shown in Figure 10A. Additionally, process recovery data for the runs was compiled and is shown in Figure 10B. The red, blue, and black plots represent donors 1, 2, and 3, respectively. However, Figure 10B includes additional data points from further runs to better showcase process recovery between different donors. The data shows that high isolation efficiency was achieved on day 0. Process recovery on day 2 varied depending on the donors that were used. The average cell recovery from the start of the day 2 process prior to debeading through to the end of the day 2 process post-Xenon electroporation was approximately 60%.

Figure 11 shows post-genome engineering data, with Figure 11A depicting editing efficiency. Editing efficiency was assessed on day 5 of the process (i.e., 3 days post-electroporation) and also at the end of process, on day 12 (i.e., 10 days post-electroporation). High knockout efficiency was observed for all donors at both time points. High percentage

CAR expression was observed on the day of harvest (20%, 50%, and 83% CAR expression for the three donors tested). However, lower knock-in efficiency was noted at the earlier time points.

It seems that for at least two donors in this experiment, this process favors enrichment of CAR expression in cells post-gene modification. Figure 11B shows that the total number of CAR-T cells generated by day 12 (i.e., following cell expansion) was approximately 800,000 CAR-T cells from the donor 1 sample, 3.1 billion from the donor 2 sample, and 4.9 billion from the donor 3 sample.

A key factor that determines CAR-T cell product efficacy is retention of T cells with younger and more stem-like phenotypes in the final product. These younger phenotypes are correlated with improved clinical outcomes compared with those with differentiated memory effector function or exhaustion phenotypes, all of which are signatures of more differentiated T-cell types. Therefore, data was gathered on stem memory (TSCM), central memory (TcM), effector memory (TeM), and T effector (Teff) T cell phenotypes in order to better characterize the final CAR-T cell product generated through this process. CD45RA and CCR7 markers were studied to define the memory phenotype composition in the final product. The results are shown in Figure 11C. A reduction in the TSCM population post-genome modification when compared to the starting material or the unedited controls can be seen. However, the harvested product on day 12 is still predominantly composed of TSCM central memory cells, which is the desired population. Additionally, a very low percentage of T effector memory cells and T effector cells was found the final product, which is also desirable.

Turning to the efficacy of the final CAR-T cell product, this was measured using a cytotoxicity assay. 10 CAR-T cells were exposed to CD19-expressing Nalm6 cells for 5 h. Highly efficient target cell killing was observed (Figure 11C), demonstrating the effectiveness of the final CAR-T cell product generated through this process.

FIGURE 11

Process performance post-electroporation—genome engineering efficiency; total edited cell count; memory T cell phenotype, and CAR-T cell cytotoxicity.

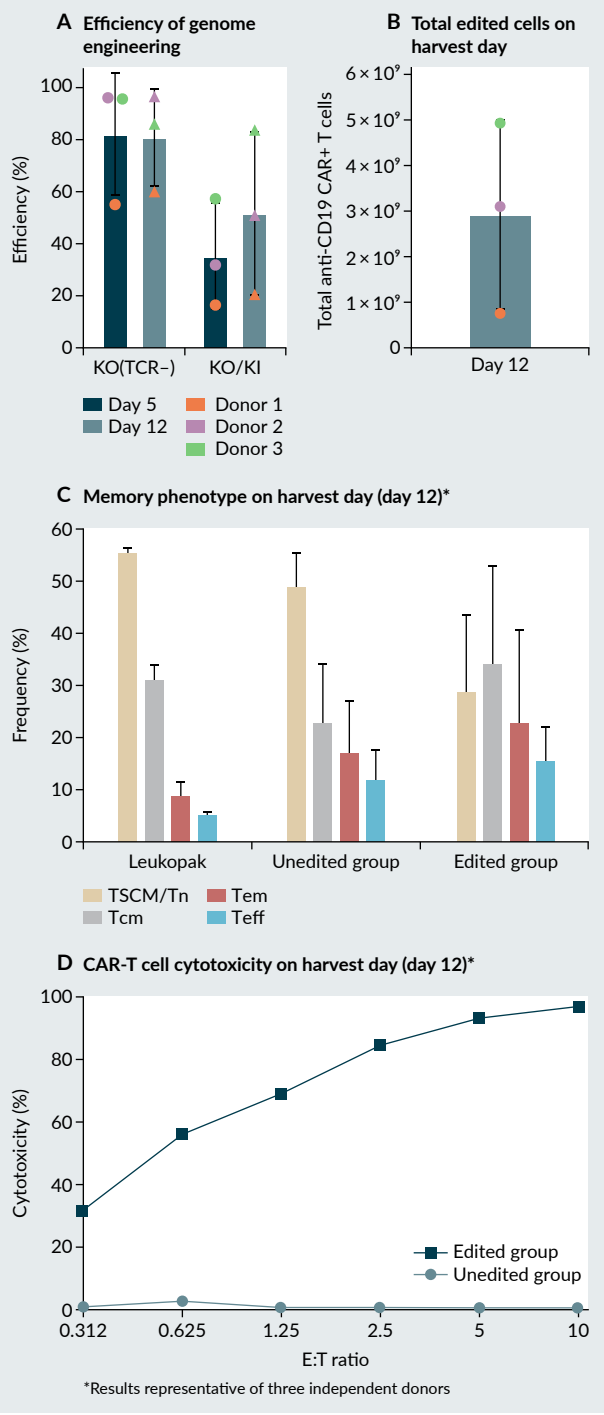
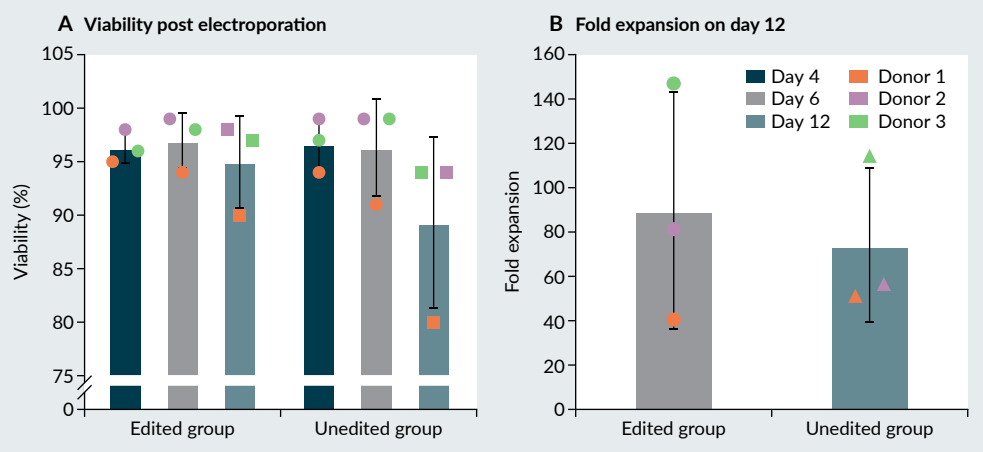


FIGURE 12

Process performance post-electroporation—CAR-T cell viability and cell expansion.



Finally, both the viability of the CAR-T cells and their expansion post-electroporation were studied. Figure 12A shows the viability on days 4, 6, and 12 of the process (i.e., days 2, 4, and 10 post-electroporation, respectively). Viability remains high post-electroporation, although a slight drop below 90% for one of the donors was observed on the day of harvest. High fold expansion in static culture of edited T cells was observed (Figure 12B). Interestingly, for two of the donors used for these experiments, better expansion of edited T cells was seen compared to the unedited control. This would again suggest that this process can help enrichment of edited T cells or CAR-T cells during cell expansion. The reasons behind this enrichment are currently being studied.

In summary, an end-to-end non-viral CAR-T manufacturing workflow using HiFi Cas9/Genome editing buffer and the large-scale CTS Xenon electroporation system was studied. High process recovery for both day 0 isolation and day 2 genome engineering steps of the workflow was observed. T cell purity was very high prior to electroporation, while high knockout/knock-in efficiency was seen post-electroporation. Although off-target data for large-scale experiments is not discussed here, no off-targets effects were found in the end-to-end non-viral CAR-T workflow when HiFi Cas9 was utilized for genome engineering. The final product generated through the process showed high cytotoxicity when CAR-T cells were exposed to CD19-expressing Nalm6 cells within a 5 hour timeframe. There was high enrichment of early memory phenotype cells (TSCM central memory cells) in the final product generated through the process.

Ongoing work at Thermo Fisher Scientific includes the optimization of non-viral CAR-T workflows with the aim of streamlining this process. One current focus here is the investigation of culture conditions that enable improved expansion of CAR-T cells at earlier time points post-genome modification, with the purpose of more quickly reaching a required dose and thus, shortening this non-viral CAR-T cell workflow. Additionally, more automated approaches for associated product characterization are being developed.

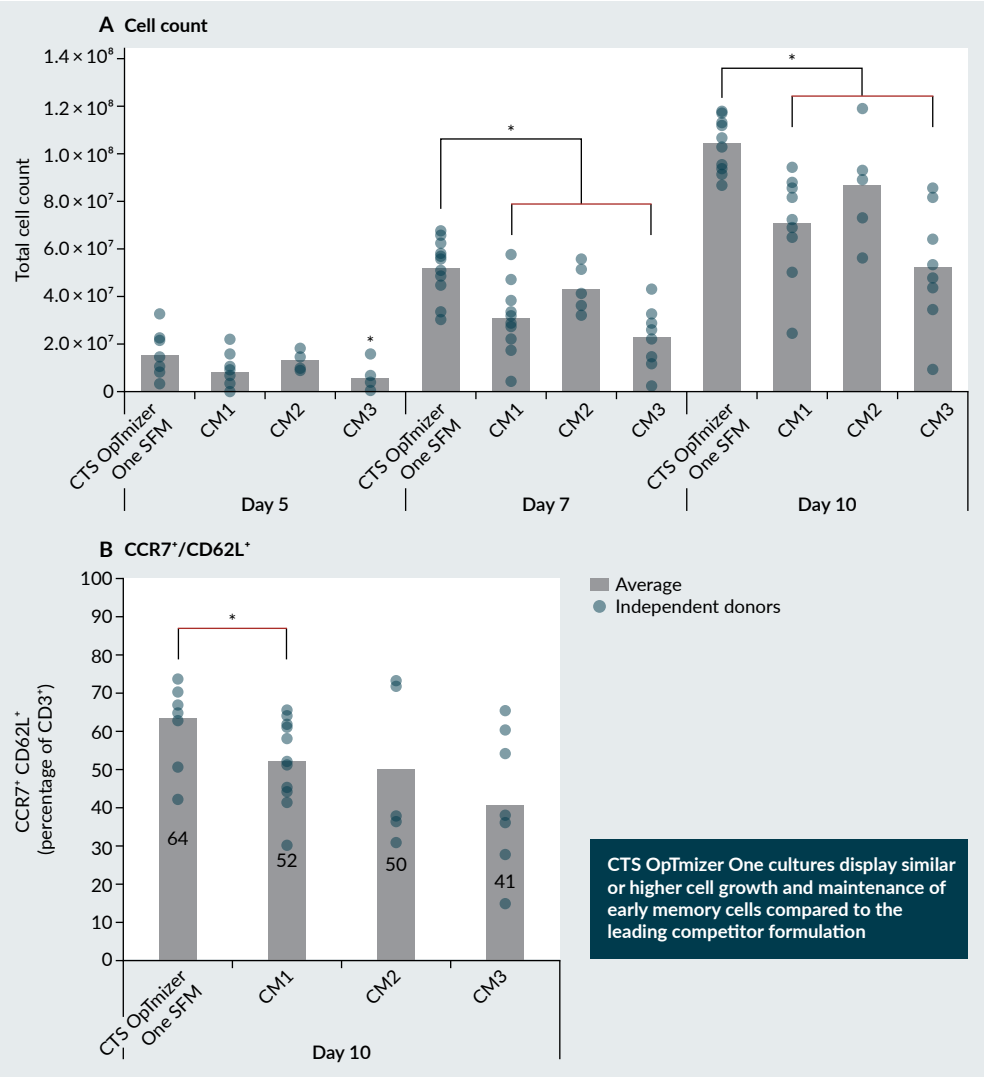
SCALABLE CAR-T CELL EXPANSION

A major contributing factor in the CAR-T cell expansion phase is the culture media: it touches and nourishes the cells at every step of the manufacturing workflow and has a significant influence over the final cellular product.

CTS™ OpTmizer™ One SFM is a new medium that is designed to improve

FIGURE 13

Comparative cell expansion performance and early memory cell phenotype maintenance of OpTmizer One SFM versus other available media.

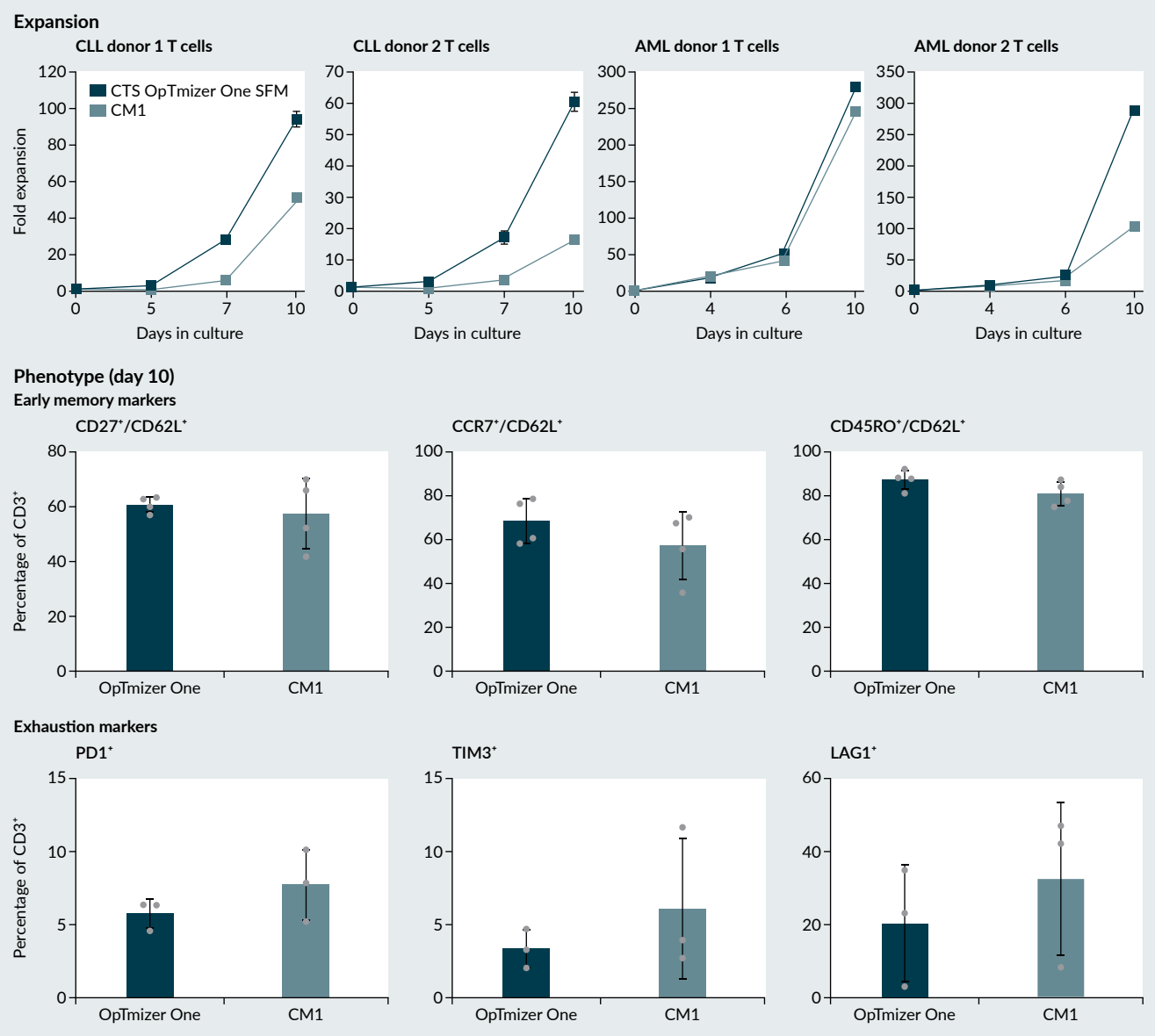


consistency and reduce risk in CAR-T cell expansion processes. OpTmizer One SFM is a one-part, animal-origin-free (AOF) formulation, which, despite its name, is not a derivative of the other OpTmizer media products. Instead, it was designed using advanced analytical tools to encourage cellular fitness and a more youthful phenotype and function. Beyond the media itself, OpTmizer One SFM is equipped with packaging designed specifically with closed, automated, and scalable workflows in mind, serving both to reduce the number of manual touchpoints in the manufacturing process and improve the safety and cost profiles of CAR-T cell therapy manufacturing. The fact that it is part of the CTS family of products also means that OpTmizer One SFM comes with regulatory support and documentation for seamless clinical application.

A recent study compared the OpTmizer One SFM with two other popular animal origin-free media formulations and one xeno-free formulation. Figure 13 shows data relating to cell expansion and phenotype capabilities. The cells were activated with Dynabeads CD3/C28 magnetic beads and cultured in G-Rex vessels. In Figure 13A, cell expansion data is plotted as total number of cells on days 5, 7, and 10. The

FIGURE 14

Comparative cell expansion performance, early memory cell phenotype maintenance, and exhaustion markers for OpTmizer One SFM versus the leading competitor AOF medium in diseased donor cells.



dots represent individual donors, while the gray bars show the average of all donors. The data demonstrates that OpTmizer One SFM delivered higher cell counts at all time points compared to the other media tested. On average, an approximate 200-fold expansion at day 10 was achieved, with the narrow range of results showing that this was consistent across all donors.

It is often the case that higher growth correlates with greater differentiation. However, Figure 13B shows that co-expression of CCR7 and L-selectin (CD62L) remained significantly higher in the OpTmizer One SFM group. Overall, greater growth and a higher percentage of early memory T cell phenotypes were observed. Additionally, cell viability was comparably high across all media (data not shown).

The next step was to validate these results with disease donor cultures. Cells from two donors with chronic lymphocytic leukemia

(CLL) and two donors with acute myeloid leukemia (AML) were cultured just as in the previous experiment. The cells were expanded in either OpTmizer One SFM or the leading competitor AOF medium. Figure 14 shows the fold expansion for each individual donor. In all cases, OpTmizer One SFM expanded cells as well as or significantly better than the leading AOF medium. Three of the donors showed particularly stark improvement with OpTmizer One SFM.

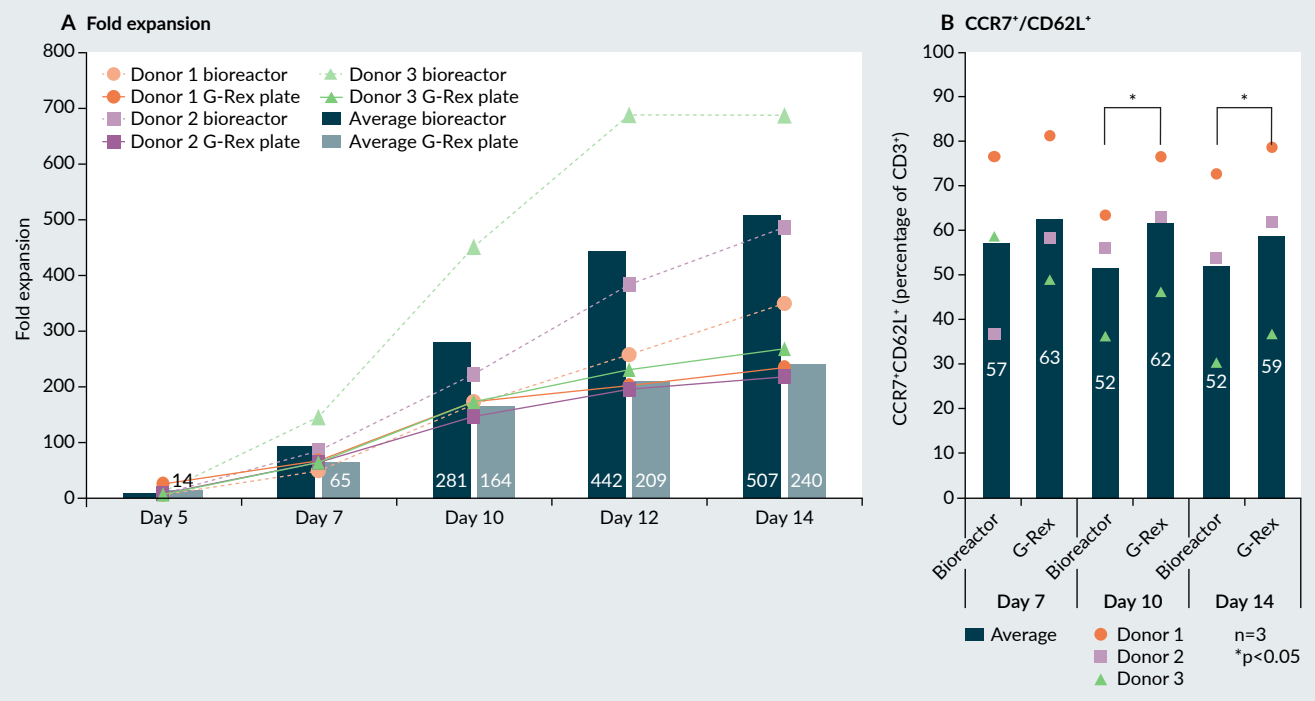
Phenotype data is also shown, including both early memory marker expression and exhaustion marker expression. Expression of early memory markers was as high or higher in OpTmizer One SFM, while exhaustion markers were consistently as low or lower than the other AOF medium. Therefore, as was the case with the healthy donors, the diseased donor cells cultured in OpTmizer One SFM showed improved growth and phenotype versus the leading competitor AOF medium.

The scalability of OpTmizer One SFM was also assessed in rocking motion bioreactors. T cells from 3 donors were activated with Dynabeads before being cultured in both a perfusion rocking motion bioreactor and G-Rex vessels, the latter using a process that mimics perfusion via exchange of 80% of the vessel volume on a daily basis. OpTmizer One SFM performed very strongly in the scale-up bioreactor, showing enhanced proliferation at all time points compared to the static G-Rex vessel. Figure 15 shows that at day 10, there was an approximately 300-fold expansion on average, which represented about a 70% improvement on the G-Rex. This result underscores the promise that OpTmizer One SFM shows in larger-scale, more dynamic bioreactor settings.

Figure 15 also shows early memory marker expression data across the different vessels, with a slight trend in favor of the G-Rex observed. However, the large difference in expansion far outweighed that in marker expression. The relative number of desirable cells at each step of the culturing process was also considerably higher in the rocker

FIGURE 15

Assessing OpTmizer One SFM performance in a scale-up setting utilizing a rocker bioreactor with perfusion.



bioreactor. Once again, viability was high in both vessels (data not shown), but it was more consistently higher in the bioreactor. This was expected as perfusion does tend to nourish higher cell densities particularly well.

Beyond simply growing cells, another critical requirement for cell therapy media is the ability to support both virally and non-virally engineered cells. This is consistently a challenge for engineered cell therapy developers; consequently, it was a focal point for Thermo

FIGURE 16

Comparative transduction efficiency and subsequent transduced cell expansion performance.

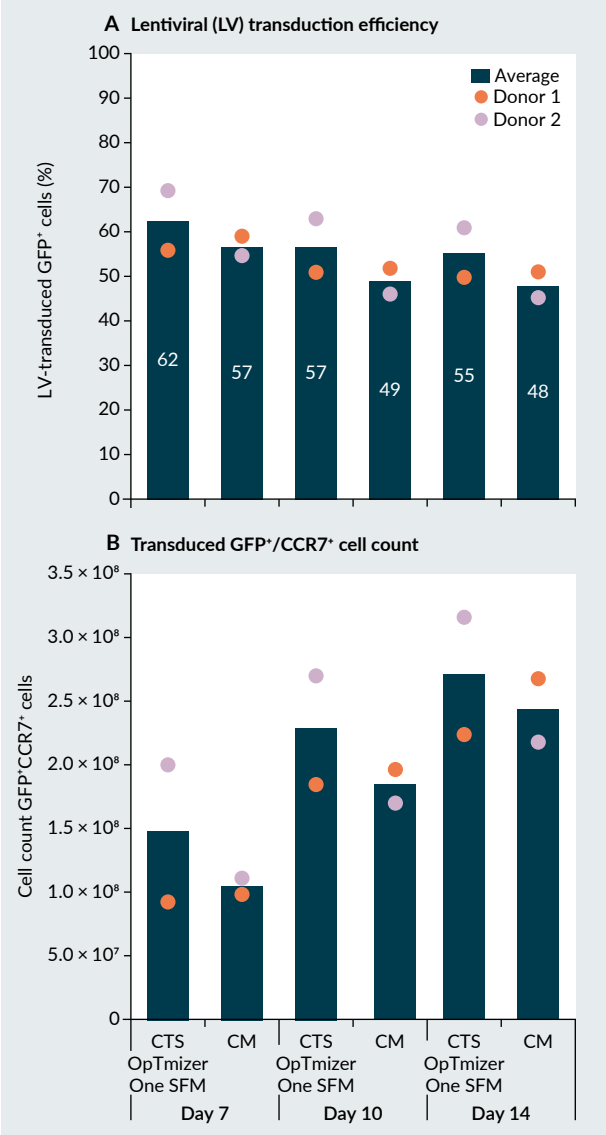
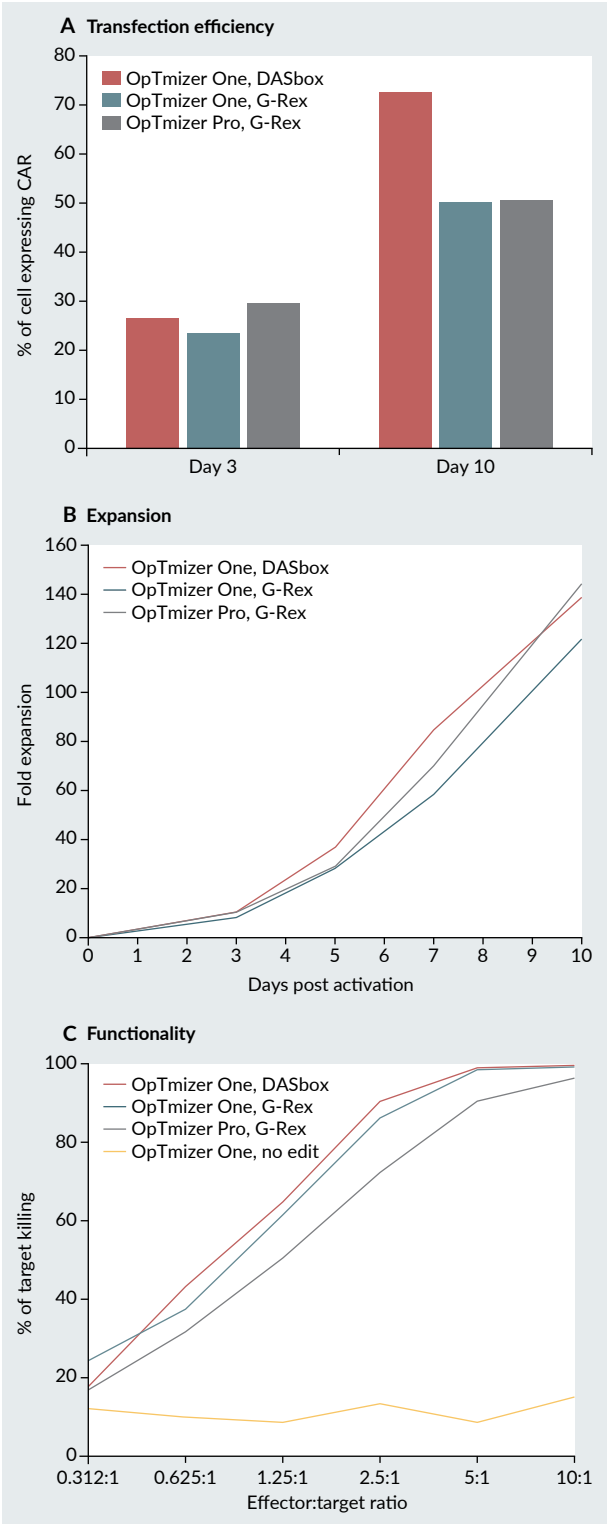


FIGURE 17

Comparative transfection efficiency and subsequent transfected cell expansion performance and target cell killing functionality.



Fisher Scientific during the development of OpTmizer One SFM.

A study of transduction was conducted by an early external tester of OpTmizer One SFM. Here, T cells from two donors were transduced with GFP lentiviral vectors. The cells were expanded in OpTmizer One SFM and in the leading AOF medium. Quantification of the GFP expression demonstrated comparable or slightly higher expression for the cells cultured in OpTmizer One SFM at all time points during the process (Figure 16). This figure also shows that OpTmizer One SFM was associated with similar or enhanced growth over the full time period. This experiment has since been repeated on multiple occasions with CAR in perfusion bioreactors, with similar results. Again, highly comparable viability across both media tested was observed throughout the workflow (data not shown). In conclusion, OpTmizer One SFM is highly effective in supporting transduction and the subsequent expansion of transduced cells.

Turning to non-viral engineering, transfection is generally more of a challenge for cell therapy developers, being considerably harsher on the cells. On this occasion, OpTmizer One SFM was directly compared to OpTmizer Pro SFM. While additional experiments have been conducted comparing OpTmizer One SFM with other available AOF media in this particular setting, this was an experiment where a completely closed non-viral workflow was employed utilizing the CTS DynaCellect, Xenon, and Rotea systems, and either fed-batch stirred-tank bioreactors or G-Rex vessels. The resulting data in Figure 17 shows healthy, comparable levels of knock-in on day 3 of approximately 25–30%. By day 10, enrichment of the CAR$^+$ T cell population may be seen in the G-Rex vessels, but not to the same extent as in the stirred-tank bioreactors. This is a further example of a consistent effect with these media observed across multiple sites and experiments. Figure 17 also shows that this effect is not merely associated with the non-edited cells simply dying off which may shift the ratio. In conclusion, alongside the strong transfection efficiency, OpTmizer One SFM supported high growth of cells post-electroporation, which resulted in an approximate 120–140-fold expansion by day 10—a robust result.

Taken together, the comparable growth with enrichment of additive cells equates to considerably more of the all-important CAR-expressing cells. In the final graph in Figure 17 (function) shows the results of the cell killing assay used in this experiment. A very high killing capacity is demonstrated across all of the conditions, media, and vessels, but both of the OpTmizer One SFM conditions displayed a 10 to 15% increase in target killing along the dose curve over the OpTmizer Pro SFM.

In summary, OpTmizer One SFM is a new AOF formulation that was designed to improve growth of desirable T cell phenotypes. OpTmizer One SFM encourages efficient CAR knock-in together with robust growth and enrichment of highly functional genetically engineered cells. It has packaging that was designed with convenience and flexibility in mind and can be readily integrated into closed and automated systems to reduce the number of manual touchpoints in the process.

Q&A



Mina Ahmadi, Jason Isaacson, and Evan Zynda (pictured left to right)

Q How does the CTS Detachable Dynabeads technology enhance the efficiency of T cell manufacturing compared to traditional methods?

JI: First and foremost, Detachable Dynabeads are available as a Gibco CTS product, which means they can be taken directly through into manufacturing without any issues. Of course, they also allow you to both isolate and activate your cells at the same time, reducing the need for a secondary activation reagent. Finally, what differentiates Detachable Dynabeads from traditional passive release beads is that the release buffer may be used to remove the Dynabeads at any desired time point in the process day. This allows for tunable activation exposure to fit a specific workflow process.

Q If I need to have a specific CD4/CD8 ratio for my final drug product, would this process still work?

MA: We have CD4/CD8 beads in the pipeline, which will hopefully be launched in the near future. They are being developed using the same detachable bead technology discussed above. If the CD4/CD8 ratio is important in the final product, these CD4/CD8 detachable beads could be leveraged to isolate CD4/CD8 T cells separately, and then the CD3/CD28 detachable beads could be used for their separate activation. It would then be possible to employ the same genome modification step or process discussed in the article above. The CD4 and CD8 cells can be cultured separately and then, at the end of process, mixed together in the desired ratio for the final product.

Q What are the considerations associated with implementing automation in CAR-T cell manufacturing processes?

EZ: To me, the three major challenges facing the CAR-T cell therapy field today are efficacy, cost, and safety—in particular, as all three pertain to patient access and availability to a wider patient population. Automation can address all of these challenges in some form.

The most direct relationship is with safety. Automation can reduce manual touchpoints, limit contamination, and reduce potential product failure modes that can completely derail cell therapy application. Regarding cost, with the reduction in manual touchpoints comes a reduced need for specialized labor—this obviously reduces the costs considerably. Efficacy is perhaps a bit more of a reach, but by automating everything, one may achieve a more consistent and robust process, which can in turn lead to a more patient-/donor cell-agnostic outcome—in other words, you can always achieve the most effective outcome regardless of what the input cell looks like.

REFERENCES

1. Naim S, Smith J, Yang G. Driving the next wave of innovation in CAR T-cell therapies. *McKinsey & Company* 2019. https://www.mckinsey.com/industries/life-sciences/our-insights/driving-the-next-wave-of-innovation-in-car-t-cell-therapies
2. Patient Access Analytics. *Oribiotech* 2024. https://oribiotech.com/news/patient-access-analytics
3. Tomtishen J. The role of automation in meeting the growing demand for CAR T-cell therapies. *Am. J. Manag. Care* 2023; 29(5), SP427—SP428.

BIOGRAPHIES

MINA AHMADI is an R&D lead scientist at Thermo Fisher Scientific, San Diego, CA, USA where she focuses on developing end-to-end closed and automated viral and non-viral cell therapy workflows. Dr Ahmadi received her PhD in Molecular Genetics from McGill University, Montreal, Quebec, Canada. After her PhD, she joined as Postdoctoral Fellow at the Department of Pharmacy, University of Toronto. Her group supported regulatory submissions including IND, NDA, and CMC updates, regulatory inspections, NC/CAPA investigations, contamination investigations and remediation, and developed regulatory strategy for implementation of new methods.

JASON ISAACSON is a scientist in the Cell and Gene Therapy business on the Cell Therapy Innovations team at Thermo Fisher Scientific, San Diego, CA, USA. He is responsible for development and optimization of end-to-end cell therapy workflow solutions that integrate Thermo Fisher's closed, automated instrumentation platforms. Prior to this role, Jason has gained experience across many industrial environments from large pharma, to small biotech and startups. His extensive experience in research includes 10 years developing HIV small molecule inhibitors, 7 years developing small molecule inhibitors for various oncology targets, and 7 years working with T cell and natural killer cell therapy development.

RUSSELL JARRES spent a decade at the bench in a variety of biotech roles doing drug discovery, analytical chemistry, and diagnostic development before transitioning into marketing and commercial roles for the last 11 years. He was previously the Senior Technical Specialist for Stem Cells/Regenerative Medicine at Thermo Fisher Scientific, Manchester, NH, USA before joining the Cell and Gene Therapy team as the Senior Manager for Strategic Collaborations. In this role, he works to connect developers with Cell Therapy Innovations (CTI) partners across Thermo Fisher Scientific to accelerate CGT programs externally. This includes access to a dedicated team of process development scientists who will adopt customer processes onto our closed and automated workflows, provide early access to innovations from Thermo Fisher Scientific's R&D teams, and support across all divisions to complete Thermo Fisher Scientific's commitment from innovation to clinic.

EVAN ZYNDA has been with Thermo Fisher Scientific, Grand Island, NY, USA for almost 8 years. He serves as a Senior Scientist in R&D for the department of Cell Culture and Cellular Medicine, and the align team in cell therapy process development and product development. He first began studying T cell biology in 2005 at Roswell Park Cancer Institute, Buffalo, NY, USA where he received a PhD in Molecular and Cellular Biophysics and Biochemistry. During his academic years, he elucidated mechanisms by which tumor cells evade the immune system. He went on to apply this knowledge in drug development and cell therapy manufacturing.

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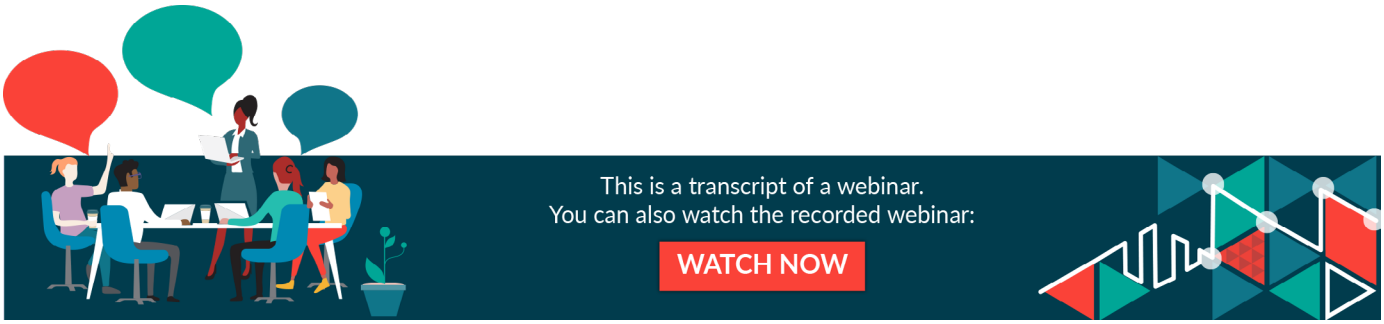

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WEBINAR DIGEST

Evaluation and performance of an AAV affinity resin

Buzz Lobbezoo, Field Application Scientist, Thermo Fisher Scientific, Cambridge, UK

Growing demand for high-quality AAV vectors for clinical applications is driving a need for the development of increasingly efficient and robust downstream purification processes. The performance of the affinity capture step of AAV as a scalable unit operation is of particular importance to deliver high purity and recovery.

Designed for improved process performance and productivity for a wide range of biomolecules, POROS™ CaptureSelect™ affinity resins allow for linear pressure drop versus flow curves up to high pressures and flow rates. In addition, the pore structure of the POROS bead enables efficient purification of large molecules. The CaptureSelect technology platform is highly selective for AAV serotypes, with the POROS CaptureSelect AAVX resin offering affinity to a wide range of serotypes, including both natural and chimeric vectors, and ensuring high binding capacity, purity, and recovery.

Figure 2. POROS CaptureSelect AAVX elution optimization study (data generated by Thermo Fisher Scientific).

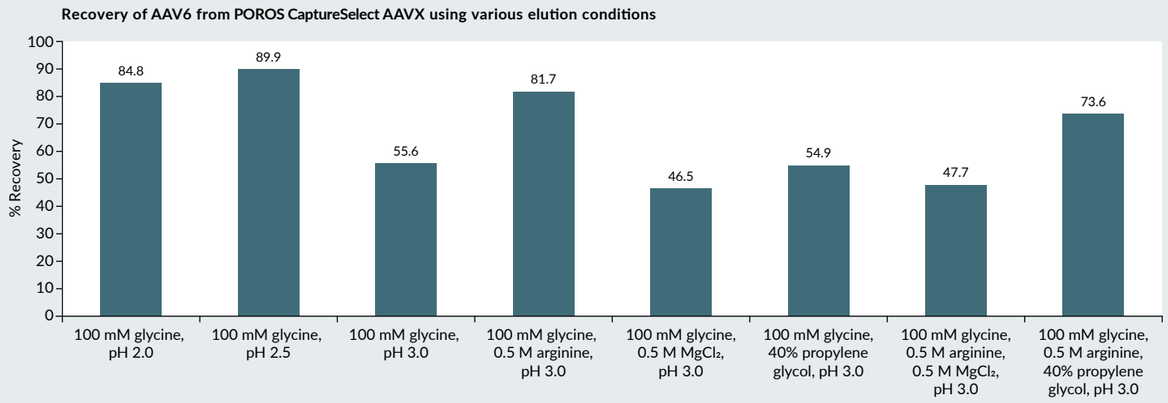
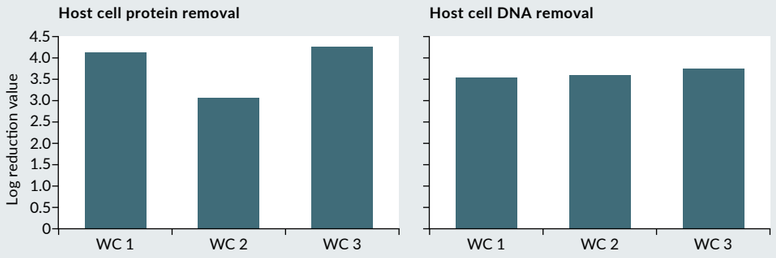


Figure 1. POROS CaptureSelect AAVX impurity removal (data generated by Thermo Fisher Scientific).



Wash	Intermediate washes
All combinations included a primary wash step of 50 mM Tris, 1.5 M NaCl pH 9.0	
Combination 1	50 mM Tris pH 7.5
Combination 2	50 mM citrate, 250 mM arginine pH 5.5
Combination 3	50 mM Tris pH 9.0

*HCP and DNA levels in the elution were ~4× higher without the washes; WC=Wash combination.

The POROS™ CaptureSelect™ AAVX resin is serotype-specific, so process development is critical to obtain optimal performance. Intermediate wash optimization has been shown to improve the clearance of process-related impurities. As shown in **Figure 1**, a wash study was performed to remove additional non-specific DNA and host cell protein (HCP) binding employing low and high concentrations of NaCl washes with no salt and 1.5 M salt, and an increased pH wash at pH 9.0. Following the wash step, levels of residual DNA and HCP were reduced 4-fold in the elution.

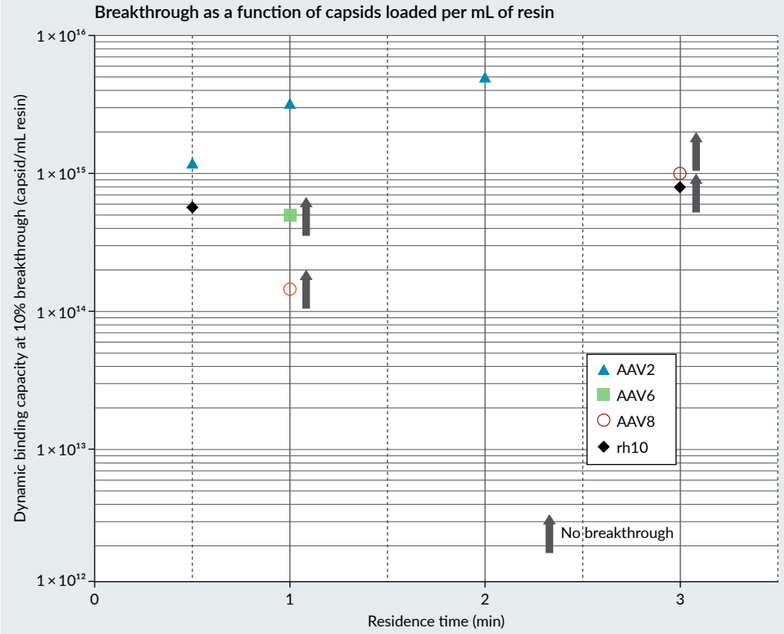
Optimization of elution conditions is also required to maximize AAV recovery. **Figure 2** shows the recovery of AAV6 from POROS CaptureSelect AAVX resin using various elution conditions. Additives can be used in the elution buffer to improve recoveries at higher pH conditions.

Analytics also play an important role in ensuring accurate results. One important feature of POROS CaptureSelect AAVX is its high binding capacity for multiple serotypes at short residence times (**Figure 3**). Capacities have been shown to exceed 1×10^{15} viral capsids (vp)/mL of resin, achievable with no breakthrough in the case of AAV8. POROS CaptureSelect AAVX also gives consistent chromatographic performance and yields over 35 reuse cycles.

Watch the webinar here

Read the full transcript here

Figure 3. POROS CaptureSelect AAVX dynamic binding capacity (data generated by Thermo Fisher Scientific).



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