Biotechnology Reports 37 (2023) e00784

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Review

Characterization of human induced pluripotent stems cells: Current approaches, challenges, and future solutions

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A R T I C L E I N F O

Keywords:
iPSCs
Cell therapies
Analytical methods
Assay robustness
Automation

A B S T R A C T

Human induced pluripotent stem cells (iPSC) have demonstrated massive potentials for use in regenerative and personalized medicine due to their ability to expand in culture and differentiate into specialized cells with therapeutic benefits. However, in order to industrialize iPSC-derived therapies, it is necessary to address the existing challenges surrounding the analytics implemented in the manufacturing process to evaluate and monitor cell expansion, differentiation, and quality of the final products. Here, we review some of the key analytical methods used as part of identity, potency, or safety for in-process or final product release testing and highlighted the challenges and potential solutions for consideration in the Chemistry, Manufacturing and Controls (CMC) strategy for iPSC-based therapies.

Some of the challenges associated with characterization and testing of iPSC-based products are related to the choice of analytical technology (to ensure fit-for-purpose), assay reliability and robustness. Automation of analytical methods may be required to reduce hands on time, and improve reliability of the methods through reducing assay variability. Indeed, we have shown that automation of analytical methods is feasible (evaluated using an ELISA based assay) and would result in more precise measurements (demonstrated by lower co-efficient of Variation and standard deviation), less hands-on time, and swift compared to a manually run assay. Therefore, in order to support commercialization of iPSC-based therapies we suggest a well-designed testing strategy to be established in the development phase while incorporating robust, reproducible, reliable, and potentially automated analytics in the manufacturing process.

1. Introduction

Human Induced Pluripotent Stem Cells have the potential to change the way we approach the treatment of chronic disease and acute traumatic injury through their ability to be differentiated into any type of cell and tissue within the body. These treatment paradigms, and their development, rely on a well characterized and controlled process for the manufacture of the starting material, namely the iPSCs themselves. In brief, the most commonly encountered allogeneic iPSC process begins with reprogramming of somatic cells isolated from a healthy donor, either through plasmid or viral vector based approaches, followed by selection and expansion of the resulting iPSC through serial passaging in two dimensional (2D) cell culture vessels or three dimensional (3D) cell culture systems. Once generated and expanded at appropriate scale, the iPSC need to be differentiated into therapeutically relevant cells under controlled conditions. Therefore, to unlock the true potential of iPSC for therapeutic applications, it is necessary to establish a robust, reproducible, well characterized, and cGMP-compliant manufacturing process covering generation and / or expansion of starting materials (i.e. iPSC), followed by directed differentiation into specialized cells.

Considering the complexity of reprograming and differentiation processes, the development of manufacturing process for iPSC-derived cell therapy products relies heavily on performing accurate measurements, compilation of the defining data, and predicting the biological status of pluripotent stem cells, or their progenies, in every step of the process. The establishment of the correct suite of release and characterization assays is necessary to control and monitor the identity, purity, safety, and potential use of the starting materials, iPSC themselves.

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https://doi.org/10.1016/j.btre.2023.e00784
Received 9 August 2022; Received in revised form 9 January 2023; Accepted 19 January 2023
Available online 24 January 2023

Although some common release assays may have been used for release of iPSC, the assays used for detailed characterization differ based on the target cell therapy indication and the laboratory or manufacturing organization. While the path to the correct and appropriate analytical characterization appears to be more clear for autologous iPSC-based therapies [1], the regulatory guidelines for derivation and manufacturing of allogeneic iPSC-based products are evolving. It may be possible to consider allogeneic iPSC banks more akin to unfinished intermediate material (or starting materials) in the development and manufacturing of the respective Cell Therapy product. With this perspective, the proper identification and use of appropriate assays to demonstrate the identity, safety, and potency/ potential of use of these banks could be problematic.

Though previously manufactured iPSC banks have been through a litany of characterization assays, the assays necessary for their GMP release will likely not be as comprehensive. Assays including whole genome sequencing, microarray, RNAseq, and DNA Methylation [2–4] were used to characterize iPSC banks, though their applicability to routine testing is unlikely. In addition, the decision of the appropriate suite of assays for release can be compounded by the final use, state and goal of the institution that manufactures them. In the past, we have argued that established iPSC master cell banks undergone comprehensive testing and characterization methods may be used for the manufacturing of multiple iPSC-derived cell therapy products [2,5,6]. Even if due to licensing or commercialization reasons, other groups wished to generate new iPSC lines, the same manufacturing process along with the library of analytical methods and characterization assays can be used for these new lines [4].

Some of the key considerations for the choice of assays used for manufacturing of allogeneic iPSC-based CT products include (1) purpose of assay (i.e. what information is necessary), (2) specification or target criteria, (3) reproducibility and assay robustness. In addition, the use of allogeneic batches is also burdened by the questions of genetic stability, in particular long term stability of the master cell banks (MCB) or working cell banks (WCB), as iPSC go through multiple freeze / thaw and serial subculturing in different cell culture formats [5].

In order to establish a robust and commercially viable GMP compliant process, it is critical to identify the critical quality attributes (CQA) in relationship with the critical process parameters (CPP). To accomplish this goal for the iPSC manufacturing process, a library of analytical methods is required, considering a set of release testing focused on identity, safety, purity, and viability of the intermediate or final products, as well as additional characterization assays intended to generate a database to monitor changes in the cell characteristics of phenotype in culture [4,7]. Certain end users may request additional specifications tied to the manufactured batch to demonstrate fit for purpose of the bank. Furthermore, the additional characterization assays may be considered as For Information Only (FIO), intended use as in-process monitoring or a better understanding of the batch that was produced (Fig. 1 and Table 1).

Analytical methods are required not only for quality control (QC) of the end product but also for characterization of the manufacturing process at early clinical and eventually commercial phases. As the Cell Therapy field expands, the industrialization of the highly manual iPSC generation process largely depends on the implementation of highly robust and reliable analytical methods in the manufacturing process. In particular, the next generation iPSC processes will need to incorporate automated workstreams that integrate automated analytics with either an automated expansion or reactor-based cell culturing system. The interaction between in-process analytics, control strategy, and automation will be an exciting development in the field. In this manuscript, we aim to highlight the gaps and challenges in the current assays used for the release and characterization of iPSC, define whether assays are necessary for release or FIO, challenges with the development or data analysis of new generation of assays, the need for automated analytical methods, and how further integrating analytics into the iPSC manufacturing process can assist faster product release and identify the next-generation assays for product characterization.

Table 1
Common Release and Characterization Assays: summarizes the general scope of GMP release testing, and additional characterizations applied to these iPSC banks .

Test	Purpose	Use	Reference
Sterility and adventitious agents	Safety/Sterility	Release	[2]
Mycoplasma	Safety/Sterility	Release	
Endotoxin	Safety/Sterility	Release	
Flow Cytometry	Identity/Purity	Release	
Cell count and viability (CCV)	Content	Release	
Reprogramming Clearance	Safety/Sterility	Release	
Karyotype	Safety Sterility	Release	
EB Formation	Potency	Characterization	
Directed Differentiation	Product Specific Potency	Characterization	
Telomere Analysis	Safety/Use	Characterization	[5]
Alkaline Phosphatase	Identity/Use	Characterization	[2]
HLA Characterization	Safety	Characterization	
Congenital Disease Markers	Safety	Characterization	

2. The need for iPSC assays optimization and robustness

A key driver for improvements in the quantitative analysis of the cell therapy drug product is the progress in the bioanalytical sciences, both technical and methodological. As assays are being developed, the expectation for acceptable assay performance needs to be specified based on consistent assay outcome. These specifications are established during the assay optimization phase, via a series of parameter read-throughs. The parameters that are evaluated and optimized during the optimization studies include (i) specificity, (ii) linearity, (iii) accuracy and (iv) precision. Specificity of an assay is the ability to distinguish between a positive and a negative control. For example, a Flow Cytometry assay should be able to detect a positive cell population along with the negative for the same marker. OCT4 and NANOG are essential pluripotency markers and a cell line selected for specificity will be negative for these markers. A positive control or a system suitability control shall include a cell line that is positive for OCT4 and NANOG markers, and therefore such cell lines would be specific for these iPSC markers. Linearity of an assay would identify the linear range of the assay for a given product. For instance, the linearity of a cell count and viability method is established to understand the linear range of the cell counts of a drug product. This will help establish the dilution of the product for measurement of cell counts within a linear range. In association, the accuracy will determine the range of the assay that yields acceptable product recovery and should be established across the specified range of the analytical procedure. For example, an analyte spike-in for enzyme-linked immunoassay (ELISA) should be able to accurately capture the spike-in percentage from the readout thus indicating the recovery percentage. Failure to recover the spiked-in analyte in the given range will result in an assay that is not reliable and will not be able to pass the accuracy of the study. Linearity and accuracy attributes help determine the Lower Limit of Quantification (LLOQ) and Upper Limit of Quantification (ULOQ). Precision assesses the acceptable variability of the assay, which helps understand if there is a need and routes for further assay optimization. Precision is determined by calculating the intra-assay precision (amount of variability present within an assay), inter-assay precision (amount of variability present in experiments on a single day), and intermediate-assay precision (variability in results from all days and all analysts combined). Measuring the percentage coefficient of variation (%CV) across the different days of assays would help establish the variability in the assays. For instance, the precision of the qPCR assays is expected to have low variability

(<10% CV). However, an increased variability in the assays would suggest a route for further optimization of the method to increase the assay's precision. These performance parameters are used to generate robust and reliable assays that will pass assay validation. These parameters are derived from U.S Food and Drug Administration(FDA) - International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines and our experience. Our goal is to develop robust assays that can tolerate minor changes and help sustain a higher level of productivity and efficiency early on. The robustness of an assay or method is the capacity of the assay to remain unaffected by small, but deliberate variations in methodology as described above and provides an indication of its reliability during normal usage [8]. Assessing robustness as early as possible will minimize the possibility of re-work later during the assay validation.

3. Automation of assays for release and product characterization

In sync with the era of automation and digitalization, there is a need for automation of iPSC processes and analytics as automation of the methodologies remains the key to commercialization of therapies and will enable processing of larger numbers of in-process and release samples. Any given analytical method such as enzyme-linked immunoassay (ELISA), Cell count and viability (CCV), Flow cytometry based assays, and quantitative polymerase chain reaction (qPCR) have the potential for automation. The future of the release assays has to focus on the rapid turnaround of results that, in-turn, aid quick final product release and reduce lab to patient time. In addition, the automation of FIO assays will assist faster product characterization. Figs. 1 and 3, Table 3

We have established automated procedures for several assays including CCV, ELISA, and are in the process of taking additional assays towards automation. For instance, here we report feasibility of automating ELISA-based assays using the Tecan Freedom Evo technology, a robot that is equipped with performing assays in a fast, reliable and efficient way. A comparative study between manual versus automated ELISA assay exhibited better precision with decreased%CV (Fig. 2) for the automated method. The percentage co-efficient of variance (%CV) that is represented in the Box plot showing the distribution of data demonstrates that a bulk of measured CV values lie on the lower end for automated method, confirming that the automation variability is lower than the manual variability. The absorbance reading (OD values) of most standards were similar between the runs, indicating that the ELISA raw data does not change between an automated and manual execution, whereas the manual executions has higher variability. Moreover, this comparison study showed that automation could reduce hands-on time (HOT) and save the number of resources needed to execute the assay as the average HOT was reduced from 6.5 h (of the manual time) to 3 h using automation (Table 2).

Future testing strategies will combine some of these techniques using automation, to better understand the potential and functional characteristics of iPSC. This will eventually generate new characterization assays for clinically relevant iPSC products. These approaches can be further applied to characterize several therapeutic modalities, from cancer immunotherapy to vaccine development via high-throughput screening assays.

4. Fit for purpose release and characterization assays

Fit for purpose method validation means the assays should be validated as appropriate for the intended use of the data and the associated regulatory requirements [9]. The assay validation process begins with choosing the right assay, followed by developing this assay into a validated method. The appropriate choice or design of assay depends on the application of the method and the limitations of the respective technology. Various types of assays can be used in the method validation process that range from the florescence detection of markers for identity, cell count viability for obtaining accurate cell counts of the initial and final differentiated drug product, residuals detection using PCR techniques, and master cell bank testing for viral contaminates using QPERT/FPERT (Fig. 2).

4.1. Flow cytometry-based identity and purity testing of iPSC

Designing appropriate flow cytometry panels depend on (i) the starting material, (ii) pluripotent stem cells generated through reprograming or established during expansion covering both iPSC and human ESCs, and (iii) directed differentiation into final cell therapy product.

Starting material: Different tissues serve as starting materials for iPSC development. Human Umbilical Cord Blood is advantageous due to its readily accessible nature, HLA reduced restriction and lower risk of alloreactivity [10,11]. Common surface molecules that are used to recognize the stemness-potential of the cells derived from starting tissues include: CD34 (identifies hematopoietic stem cells and progenitors) CD9 (leukocyte antigen), CD133 (Prominin 1), CD30 (tumor necrosis factor receptor), CD200 (MRC OX-2 antigen) and CD38 (Cyclic ADP ribose hydrolase) [12–14].

Pluripotent stem cells generated through reprogramming and expansion: The common pluripotency markers that are included in a panel for detecting stem cells prior to differentiation include OCT4,

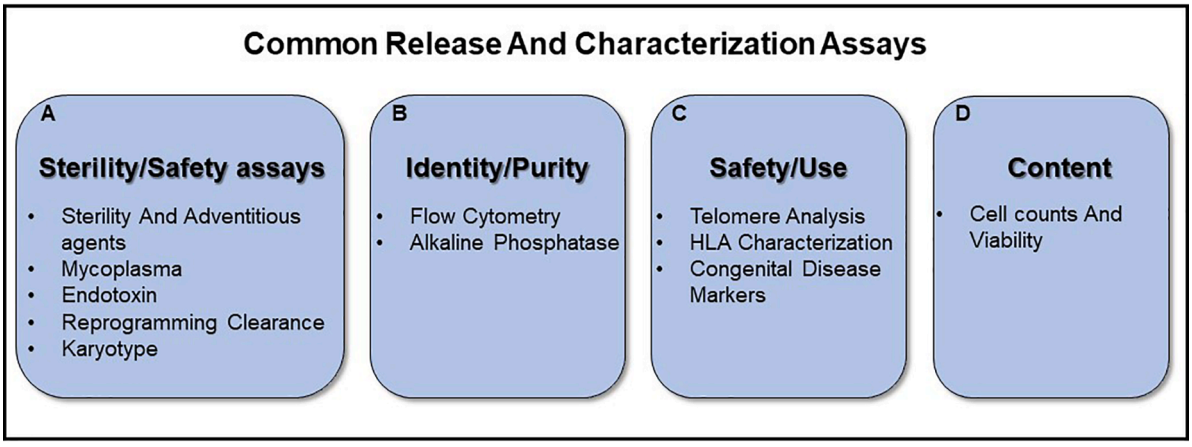

Fig. 1. Common Release and Characterization Assays: depicts the assays used for (A) sterility and safety includes sterility, mycoplasma, endotoxin, reprogramming clearance and karyotype testing. (B) identity and purity includes flow cytometry and alkaline phosphatase testing. (C) safety use includes telomere analysis, HLA characterization and congenital disease markers. (D) content release testing of iPSC banks includes cell count and viability assay.

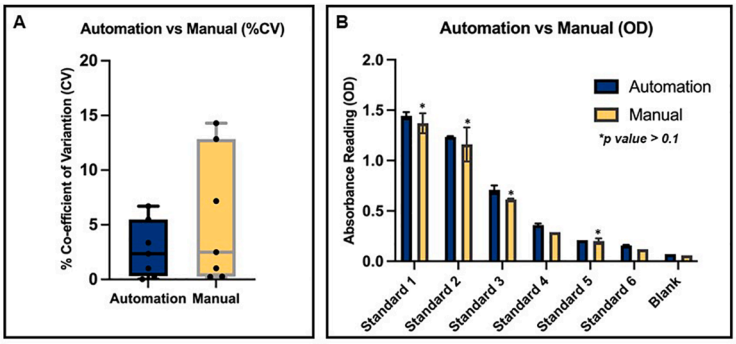

Fig. 2. (A) Co-efficient of Variation (%CV) represented in Box plot as interquartile range is lower for the Automation (Tecan) compared with manual load. (B) Absorbance reading is comparable between the Automation and Manual runs while the standard deviation in the manual run is higher compared to automation runs. Statistical method *t*-test was used to calculate the p value (*p values - Standard 1: 0.418, Standard 2: 0.599, Standard 3: 0.105, Standard 4: 0.017, Standard 5: 0.818, Standard 6: 0.035).

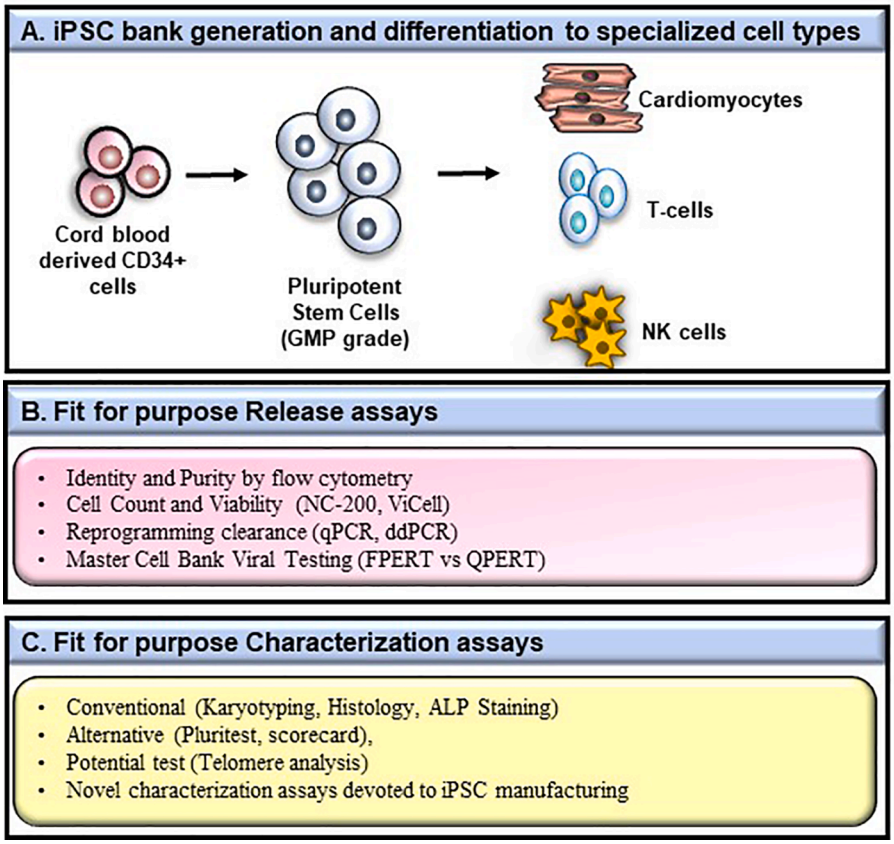

Fig. 3. (A) Illustration of iPSC cell bank generation from CD34+ cells and differentiation into cardiomyocytes, T-cells and NK-cells. (B) Proposed Release assays from the generated iPSC bank includes identity and purity by flow cytometry assay, CCV using NC-200 and ViCell, Reprogramming clearance using ddPCR and qPCR, Master Cell Bank Viral Testing using FPERT and QPERT. (C) Proposed Characterization assays includes conventional Karyotyping, Histology, ALP Staining, Alternative methods such as Pluritest and scorecard analysis, Telomere analysis as a potential test and novel assays devoted to iPSC manufacturing.

Table 2
Metric analyzed show the difference in manual and automated ELISA in terms of analyst time, assay precision and FTE.

Metric	Automation Run	Manual Run
Analyst Time	3 H	7 H
Assay Precision (%CV)	≤7%	≤20%
FTE	1.5	3

NANOG, SOX2. As iPSC undergo differentiation into specialized progenies, the differentiation process could still carry residual iPSC that need to be identified and eliminated to avoid any issues before clinical applications [15,16]. Several surface markers have been identified to distinguish iPSC that are shared with hESC. The most common ones used for recognition of iPSC are TRA-1–60, Stage Specific Embryonic Antigen 4 (SSEA4), and TRA-1–81. SSEA-4 expression seems to precede the expression of TRA-1–60 and TRA-1–80, which are only detectable at later differentiation stages (*Chan Nat Biotech 2009*). TRA-1–60 and TRA-1–80 are unique epitopes of the glycoprotein Podocalyxin and are used to identify, isolate ESCs [17].

***Targeted therapeutic cell therapy product*:** Targeted differentiation of iPSC to various final cell products have been reported including cardiomyocytes, T-cells, NK cells and B-cells for therapeutic applications. In these cases, a characterization of the final product is essential and an appropriate final product purity assay needs to be included in the release panel. For instance, cardiomyocyte products will have a flow panel identifying the purity of the final cardiomyocytes obtained from differentiation of the iPSC. This would contain mature cardiomyocytes markers such as C-troponin, calcium voltage gated channel, Myosin Light Chain and Heavy chains, and transcription factor GATA-4. Similarly a T-cell product would contain the lymphocyte markers such as CD3, CD4, CD8, and CD56 (negative marker). Furthermore, NK cell product would contain the markers such as CD16 (FcγRIII) CD57, CD56, NKG2C and likely confirm that these cells are negative for CD3 [18–20].

Table 3
List of MCB viral tests performed to test the final hiPSC bank for absence of adventitious viruses using in vitro and in vivo assays.

Assay	Type of Sample	No of cells per sample	No. of Samples	Reference
TEM	Cryo	1×10^7 cells/ml	1	[2]
Human Panel PCR	Cryo	1×10^7 cells/ml	6	
Test for Inapparent Viruses	Cell Lysate	1×10^7 cells/ml	1 × 32 ml 1 × 8 ml 1 × 3 ml 2 × 2 ml	
In vitro for presence of viral contaminants	Cell Lysate	1×10^7 cells/ml	2 × 10 ml	
Bovine Virus	Cell Lysate	1×10^7 cells/ml	1 × 12 ml	
Porcine Virus	Cell Lysate	1×10^7 cells/ml	1 × 4 ml	
PERT Assay	Supernatant	N/A	3 × 0.5 ml	
Isoenzyme	Cell pellet	1×10^7 cells/ml	1	
Adeno-Associated Virus	Cell pellet	1×10^7 cells/ml	2	
PCR (HIV, EBV, ETC) 11	Cell pellet	2×10^7 cells/ml	2 × 11 Tests	

Type of stem cells (ESC or iPSC): We and others have developed different staining panels to determine the quality of the starting material ($CD34^+$ cells from UCB), the level of expansion and purity of $CD34^+$ cells at day 4 of expansion (prior reprogramming into iPSC), and characterization of iPSC phenotype (after reprogramming and expansion) [2,4]. Final product specific flow cytometry panels have been established for a variety of cell therapy products, for instance cytoplasmic C-peptide, nuclear protein NKX6.1, insulin promoter factor PDX1 and Motor neuron and pancreas homeobox (MNX1) for insulin secreting beta cells [21], and cluster differentiation markers of lymphocytes CD7 and CD5 as T-cell progenitors and CD3, CD4, CD8, for a mature final T-cell product [22]. The flow cytometry panels for both in-process and final drug product testing undergoes rigorous optimization, a process in which the parameters such as specificity, linearity, accuracy and precision of the assay are tested to derive the product specification. A critical step during the assay optimization process is setting reliable reference controls as system suitability, for the flow cytometry assay that confirms the assay performance.

4.2. Cell counting and viability (CCV)

Cell counting and viability is an often overlooked consideration, as it seems to be a simple method, but critical step to determine an accurate number of viable cells in a given cell suspension and is an intergral part of cell line development. The most critical CCV measurement will be to test the vialing density of the final differentiated cell product. The number of cells seeded for the targetted iPSC differentiation is also to be considered as a critical parmeter to maintain the consistency of the initial feed and differentiation process. Accurate measurement of the viable cells during initiation of differentiation, cell densities at passage, cell expansion, and harvest allows better process control and eventually more accurate assessment of the amount of differentiated cells administered to a patient.

The design of an appropriate CCV assay depends on cell type, heterogeneity of the cell suspension, cell culture format (i.e. single cells or aggregated cells) [23]. Some cell types like iPSC's tend to form large colonies in 2D culture or clusters that would require enzymatic, chemical, or mechanical dissociatiation methods for serial subculturing. The individual cells in such large aggregates tend to be superimposed, reducing accuracy and precision of the cell count using a manual counting method (hemocytometer). To improve accuracy, precision, speed of the cell counts, repeatability and convenience (inbuild algorithmic features), several automated cell counter technologies (such as NucleoCounter ® NC-200, Vi-CELL Cell Viability Analyzer, Cellometer Automated Cell Counters) have been developed. The successful implementation of automated techniques depends on the adjustment of cell staining, image display parameters and cell morphology to obtain equivalent precision, accuracy and linearity with respect to the hemocytometer. For iPSC, we have shown that NC-100 and NC-200 automated cell counting devices can be used for the estimation of viable cells and viability of cells employed for iPSC platform development [2,24]. The automated NucleoCounter NC-200 (Chemometec) uses acridine orange (cell membrane) and propidium iodide (viability) while other cell counting methods such as Cellometer (Nexcelom), Vi-CELL (Beckman Coulter) uses trypan blue as the viability dye. All these automated systems can be used to assess the appropriate cell counting method for a given cell type. Accordingly, an aggregated cell protocol where in the total count is derived from separate lyzed aliquot can be used for iPSC aggregates. GMP-compliant software needs to be dedicated for the cell counting along with instrument.

4.3. Reprogramming clearance

Clearance or residual assays need to be customized depending on the reprogramming process such as plasmid transfection, transduction of plasmids using virus/ viral vectors and transposons. Reprogramming is achieved by over-expression of "Yamanaka factors" using the non-integrating episomal EBNA /OriP plasmid followed by analytical assessment of these cells for expression of Oct4, Sox2, Klf4, and c-Myc, and Lin28 [25,26]. The EBNA/OriP plasmid is commonly used for reprogramming because of its properties as a non-integrating plasmid that will clear from the cells by serial passaging [27]. The safety requirements and guidelines suggest that these cells must show full clearance of the plasmids used, illustrating the need for a fully-qualified assay that can quantify and demonstrate full plasmid clearance. One of the most reliable methods detecting residual plasmid may be TaqMan-based qPCR that allows design of primers and probes that recognize specific nucleic acid sequences with a high level of specificity and sensitivity, which will bind only to a specific DNA sequence, as opposed to Sybr-based technologies that bind all double-stranded DNA. We have previously reported using this method to amplify and measure the EBNA and OriP regions reprogramming plasmids [2]. Specificity is further evaluated by preparing the EBNA plasmid standard in carrier H9 gDNA in addition to water and analysis for Ct difference. However, there may be some challenges for proper assessment of plasmid clearance using this method. For instance, the EBNA and OriP regions are only small fragments of the larger than 1000 kb plasmids and other fragmented sections of the plasmids could remain within the cells without detection. Also, while PCR methods have a high sensitivity, the limit of detection (LOD) of any assay is rarely a perfect Zero. Therefore, more sensitive method to detect residual plasmid following reprograming may be needed.

More sensitive methods can be found in newer technologies, such as analysis by digital droplet PCR or (ddPCR), Next Generation Sequencing (NGS). Digital droplet PCR (ddPCR) utilizes the same TaqMan chemistry as many qPCR processes but offers a higher sensitivity than qPCR methods due to the ability of ddPCR to count absolute copy number [28]. Using ddPCR, the sample and PCR master mix are loaded and then formed into approx. 20,000 droplets. The droplets then undergo PCR and are read individually for fluorescence release by probes during amplification. By measuring absolute copies present, ddPCR is able to detect rare populations. However, ddPCR maintains the same risk as the qPCR that plasmid fractions may still exist within the cell. The risks of this approach can be mitigated by designing more than one primer probe set, such that multiple regions of the plasmid can be detected to insure there are no remaining plasmid fragments [28]. NGS allows for sequencing of all DNA or RNA in a sample, making it a valuable tool in

detection of residual reprograming plasmids. Use of NGS technology would allow for sequencing of the entire genome in the working cell bank selected and looking for fragments of the entire pEB-C5 or pEB-Tg plasmid rather than just the EBNA/OriP fragments. However, full-genome sequencing still remains quite costly, although there are commercially-available instruments currently on the market, such as the Illuminia MiSEQ and ABI SOLiD platforms.

4.4. Master cell bank viral testing

The release of allogeneic products as master cell banks and for clinical use requires extensive testing for the presence of viral contaminants. In the past, we demonstrated that the MCB viral testing panel could be adjusted and utilized for the release of final human iPSC banks using adequate assays and considering the cellular characteristics of pluripotent stem cells [2,6]. A variety of in vitro and in vivo analytical methods can be used to evaluate potential presence of viral contaminants in the final product including retrovirus, adeno-associated virus as well as bovine, porcine, and human viruses. Since these methods are used for release or rejection of the cell bank, they need to be robust, reliable, fit for purpose, and qualified or validated. The choice of assay used in the MCB testing panel and fit for purpose is critical for adequate evaluation of viral contaminants, because such methods are used to determine the release or rejection of the bank and could create serious safety consideration.

One of the common methods used for detection of retrovirus in the final cell bank is a PCR-enhanced reverse transcriptase (PERT) assay using the presence of an enzyme, reverse transcriptase, that drives the conversion of RNA into cDNA specifically found in retroviruses. PERT assays assess a sample ability to convert an RNA template into a cDNA template through the activity of reverse transcriptase present in the sample (i.e. supernatant). However, it has been documented that excess DNA polymerase activity in a sample can interfere and raise the background signal in these assays, which could lead to incorrect measurement of reverse transcriptase activity within the sample [29]. There are two common PERT-based assays that could be used for detection of retrovirus in cell bank including QPERT and FPERT. QPERT (Quantitative PCR-enhanced Reverse Transcriptase) assay, is a quantitative form of the reverse transcriptase assay. The observed activity from the test sample is compared to a standard curve to return a quantitative measure of the reverse transcriptase activity in the sample. These results are often returned in the form of reverse transcriptase (RT) units/mL. This assay has been designed and routinely performed (at service labs) to evaluate the RT activity as part of in-process testing strategy to evaluate retroviral level throughout the manufacturing process. An increase in RT activity throughout the process would signal a contamination event. Therefore, during initial establishment of the assay, the potential of a false positive was not of major concern. In addition, this assay is most prominently used for bioprocessing processes that utilize cell types with endogenous retroviral contamination (i.e. avian cell vaccines). FPERT (Fluorescent PCR-enhance Reverse Transcriptase) assay, is a qualitative form of the reverse transcriptase assay. Working off the same mechanism as the QPERT assay, this assay returns a qualitative read out as opposed to a specific activity readout. As a qualitative measure, the results of this assay are expressed in relation to a standard containing RT activity at the limit of detection (LOD) of the assay. This assay has been designed to assess the RT activity (and therefore Retrovirus presence) in bulk harvest and the final product of bioprocesses. Generally industry standard uses FPERT for release, QPERT for in-process.

Setting an appropriate product-specific specification based on historical data is critical in establishment of appropriate release assay for the final product certificate of analysis (CoA). In particular, depending on the qualitative versus quantitative nature of the assay, appropriate release criteria and specification must be chosen. In our hands, the presence of rapid cell division in iPSC cells, and therefore high levels of DNA polymerase activity, demands that if a quantitative assay (i.e. QPERT) is to be used for release of a cellular preparation (i.e. iPSC), an appropriate product-specific specification based on historical data should be used in the release and product CoA. For a ″Not Detected″ specification, a qualitative approach would be more suitable, such as the FPERT assay.

5. Final product characterization assays

There is a need for characterization assays/testing in order to address variability between and within the clones of stem cell preparations. This will eventually help set a specification for selecting highly functional cell lines for differentiation to desired cell lineages. Each of these characterization assays can provide different yet valuable information on the health, longevity and differentiation potential of the stem cells, which cumulatively increases the chances of selecting a high quality cell lot for the patient needs. Some of these methods used for further characterization of iPSC and iPSC-derived products are reviewed here.

5.1. Conventional methods (Karyotype/Histology/Alkaline phosphatase)

The conventional testing of pluripotency includes Karyotyping and Histology analysis. Karyotyping is one of the established techniques to monitor the genomic integrity and detect abnormalities in iPSC. The therapeutic application of iPSC rely on the stability and genomic integrity of iPSC cells. Karyotyping ensures that the chromosome number and morphology are intact during reprograming, and differentiation [30,31]. Routine monitoring of genome integrity and identifying serious alteration is very crucial for product safety in cell therapy field. Although not all the genomic abnormalities are harmful, some may have an effect on iPSC differentiation ability and may cause tumorigenesis in patients receiving iPSC-based therapies [32]. The field of Pathobiology including the detection of stem cells depended on the histological analysis of the biopsies for DNA profiling. However, this is not sustainable in the long term as the iPSC requires a more refined techniques to decipher the molecular clues, signal transduction, and accurate population detection. Utilizing multiple techniques provides a complete snapshot of iPSC genomic abnormalities. Alkaline phosphatase (AP) activity is up-regulated in pluripotent stem cells, including undifferentiated embryonic stem cells (ESCs), embryonic germ cells (EGCs), and iPSC. The Alkaline Phosphatase Live Stain maintains stem cell viability and hence this is ideal for screening colonies during early stages of the reprogramming workflow. It can also be used in later stages as a negative selection tool for identifying undifferentiated cells. We use ALP staining as a standard method for the maintenance of human iPSC and ESC to characterize the Master and Working Cell Banks under Good Manufacturing Practices conditions [2,4]. The ALP has been used an “for information only (FIO)” assay for iPSC Characterization purposes.

5.2. Established alternative methods (PluriTest, qPCR score card, Teratoscore)

Recent advancement in the field of iPSC testing have addressed the challenges inherent to this pluripotent cell type. These developments display few alternative techniques for determining the developmental potential of human Pluripotency cell lines including TeratoScore (open-source platform) [33] and ThermoFisher’s PluriTest analysis and qPCR ScoreCard [34]. The pluripotency analysis using arrays (Affymetrix, Illumina) and RNA-Seq data results can further be used for PluriTest and TeratoScore analysis. TeratoScore is an online open-source platform that distinguishes pluripotent stem cell-derived teratomas from malignant tumors. This assay is based on the teratoma formation for testing the capacity of human pluripotent stem cells to differentiate into all embryonic germ layers and translates cell potency into a quantitative measure [33]. The PluriTest is a bioinformatics assay in which the transcriptome of a test cell line is compared to the transcriptome of a large number of cell lines known to be pluripotent [35]. This test does

not directly assess differentiation capacity but can exclude cells that differ substantially from undifferentiated stem cells. An important consideration in the early stages of establishing new PSC lines is the rapid testing of a small number of cells, which can be achieved by PluriTest [36]. We have established a 3-germ layer qPCR to characterize the cell lineage [2]. There is a need for this assay to be a release assay as this gives detailed information on the gene signatures with ability to generate the three germs layers that eventually develop into all cell-types within a body. Gene expression profiling and bioinformatic quantification of such gene signatures give rise to the pluripotency scorecard assay. ScoreCard can be used for gene expression signatures to quantify differentiation efficiency [34].

5.3. Potential methods (Telomere analysis, TAT, Q-TRAP)

In addition to these established alternative techniques in testing pluripotency using scorecards, we propose using telomere measurement and activity as pluripotency read outs. Telomere regulation, maintenance and homeostasis is essential for the long-term culture of iPSC [37]. Telomerase activity and telomere length play a fundamental role in the generation and functionality of iPSC. During the reprogramming of these cells, telomerase activation and telomere length extension occur. Telomere analysis will be very useful in vitro and in vivo studies related to the quality of cell-derived products and to accurately assess the differentiation potential of stem cells and iPSC. Telomere Analysis Technology (TAT) is a Telomere length determination assay that measures the median telomere length of any cell line. TAT utilizes the high throughput Q-FISH (Quantitative - Fluorescence In-Situ Hybridization) technique [38]. Q-TRAP (Telomeric Repeat Amplification Protocol) assesses telomerase enzyme activity in whole cell lysates from blood lymphocytes and other biological samples. These two techniques of analyzing telomere length and telomerase activity in stem cells can be used as valuable indication of iPSC health and functionality and, therefore, act as soft sensor for iPSC reprograming and differentiation. This can also be used to select a manufactured cell lot that is highly functional for further differentiation activities. Accordingly, we have previously used the telomere analysis to distinguish between freshly thawed and 15 –passage-old iPSC [4,5].

5.4. Novel assays devoted to iPSC manufacturing

A novel microfluidic chip based technology for the quantification of rare hPSCs has been recently reported by Wang et.al. This method is based on stem cell quantitative cytometry that enables the ultrasensitive capture, profiling, and enumeration of trace levels of hPSCs labeled with magnetic nanoparticles in a low-cost, manufacturable microfluidic chip [39]. The microfluidic technology has been employed to detect rare hPSCs in hPSC-derived cardiomyocyte populations and can be extended to detect such rare populations in other hPSC derived products. In addition to Lonza's qPCR based residual iPSC detection [2], Kuroda et *al.* have established a sensitive assay for detection of the residual undifferentiated hiPSC in cardiomyocytes, using droplet digital PCR (ddPCR). The authors have used the ddPCR method with a probe and primers for LIN28 that significantly detected as low as 0.001% undifferentiated hiPSC in primary cardiomyocytes [40]. These novel technologies allow establishing highly accurate assays for evaluation of safety and iPSC product characterization.

Flow cytometry techniques have been commonly used for identifying various cell types including stem cells through quantification of their cell surface antigen expression using fluorescence-labeled antibodies. These methods are usually considered identity assay that also provides information on the purity of the stem cell product. Flow methods have experienced a tremendous improvement from a 2-color cell marker detection to multi-color cell specific gene markers that can even detect the internal markers. PCR based techniques have been also used to detect the cell specific genes, which can also be considered for testing purity of the stem cell product. As mentioned earlier, new PCR based technologies (e.g. droplet digital PCR) are being developed to improve the accuracy and effectiveness of measuring key characteristics of iPSC and their derivatives including purity and safety (i.e. residual impurity).

Several parameters are critical for the selection of an appropriate assay for release and characterization, including therapeutic product derived from stem cells, sample timing, target markers, and the required sensitivity of the assay. While Flow cytometry is more time- and cost-effective, PCR has a higher sensitivity. However, Flow cytometry and PCR cannot substitute each other but are advantagous if used in complimentary roles for optimizing risk stratification in clinical trials [41].

6. Conclusion and future perspectives

The promise of iPSC lies in their ability to enable the development of patient-specific, and allogenic, stem cell–based therapies. Cell based therapies are developed for several diseases including diabetes, retinal pigmentation degenerative disease and Parkinson's with on-going clinical trials from pharmaceutical companies such as Sigilon Therapeutics, Viacyte, and Vertex Pharmaceutical displaying positive outcome. An ideal iPSC process will have a zero foot print methodology utilized throughout the steps of manufacturing process (e.g. use of non-integrating mRNA for reprograming) with appropriate release and characterization assays in place, efficient, reproducible and compliant with cGMP requirements. An optimized process with analytics is expected to have high-quality, safe, and effective pluripotent stem-cell product characteristics taking into consideration the balance between quantity and quality information, cost, process invasiveness, time to results, and sample representation of the population [42]. To properly address the manufacturing challenges for commercialization of iPSC-based therapies, we propose a few initiatives including focusing on detailed iPSC characterization [4], long-term stability of cGMP compliant iPSC [6], use of computer-controlled 3D bioreactor-based manufacturing process to reduce the labor and manufacturing footprint [43], implementing proper in-process monitoring throughout the process. In addition, we have designed and established the assays that are apt for different starting materials and end product characterization, as well as, the analytics that go in-hand with the entire process of the GMP-grade iPSC generation, both in-process and release assays. Furthermore, moving towards automated analytics is feasible and can lead to reliable and accelerated results. The implementation of appropriate analytical methods using Fit-For-Purpose validation methods for in-process and final release testing as highlighted in this review is critical in order to track the critical quality attributes of the product as its being manufactured and ready for release.

Stem cell therapies hold tremendous hope in the fields of cell-based therapies, high throughput drug discovery and toxicology platforms. The scalable, closed culture systems supporting the production of large scale non-immunogenic iPSC banks combined with the automated, fit-for-purpose validated release assays can steer organ-replacement therapies. There is also a need for GMP complainant software and data analysis tools for analytical assays aimed for release of the therapeutic product. Analytical advances such as automated high throughput drug discovery, next generation sequencing, pluripotency tests and telomere analysis have helped decipher molecular pathways involved in the iPSC pluripotent state, maintenance, and differentiation. Incorporation of artificial intelligence in the existing analytical approaches will help understand the cellular behavior, associated complex gene-regulatory network, molecular clues and trajectories of differentiation pathways in iPSC.

Authors contributions

All authors were involved in the writing of the manuscript, design of the studies, generation and analysis of the relevant data. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that there are no competing interests. The authors (Sahana Suresh Babu, Haritha Duvvuru, Jillian Baker, Stephanie Switalski, Mehdi Shafa, Krishna Morgan Panchalingam, Saedeh Dadgar, Justin Beller and Behnam Ahmadian Baghbaderani) are full time employees of Lonza. The results described do not describe or endorse any commercial product.

Data availability

Data will be made available on request.

Acknowledgements

The research captured in this manuscript has been supported by funds from Lonza and /or evaluation of the previous work published in the literature. We thank Sachin Puri for support with GraphPad prism statistical software and proof-read the manuscript.

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