Doctoral Dissertation

Stability Analysis of Filling Process in Human-Induced Pluripotent Stem Cell Manufacturing

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DECLARATION

Stability Analysis of Filling Process in Human-Induced Pluripotent Stem Cell Manufacturing

"I hereby declare that this doctoral dissertation is my original work and entirely written by myself. All the sources of information which has been used here have been duly acknowledged. This doctoral dissertation has not been submitted for any degree in any institution previously"

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Adithya Nair

2023

Contents

Nomenclature

Abbreviations

hiPSC	Human induced pluripotent stem cell
BST	Binary search tree
*i*Tree	Isolation tree
SPC	Statistical process control

Parameters

X	Observation data set
x_i	Individual observation in X
n	Total number of observations in X
φ	Subset of X
t_P	Process time (h)
ρ	Spearman's rank correlation value
l	*i*Tree height limit
$h(x)$	Path length of x on an *i*Tree
$E(h(x))$	Average path length of x in an ensemble of *i*Tree
$c(\varphi)$	Average path length of unsuccessful search in BST

$s(x, \varphi)$ Isolation score of an observation x

n_c, t_P Viable cell count at t_p

n_{c0} Viable cell count at $t_P = 0$ h

$n_{c1,i}$ Viable cell count after filling in i^{th} vial

$n_{c2,i}$ Viable cell count after filling, freezing and thawing in i^{th} vial

$n_{3,i}$ Viable cell count after seeding and incubation (24 h) for i^{th} vial

Indices

V Actual volume of liquid dispensed (mL)

V^* Set value of dispensing volume (mL)

D Actual value of particle density/concentration (Particles/mL)

D^* Set value of particle density/concentration

γ Survival ratio of cells suspended in cryopreservation solution (-)

β Recovery ratio of cells after freezing and thawing (-)

α Attachment efficiency of cells after seeding (-)

P Actual cell potential

P^* Theoretical cell potential

Abstract

Chapter 1: General Introduction

The filling process is an important part of the downstream processes involved in manufacturing cell-based products, including human-induced pluripotent stem cell (hiPSC) based products. Designing the filling process for hiPSC products considering the challenges of cell manufacturability requires evaluation of process instability [1]. Since analyzing biological attributes for process stability with conventional statistical process control (SPC) is not recommended due to the limitations of parametric techniques, this study focuses on developing quality indices and a nonparametric analysis algorithm for the hiPSC filling process. The thesis aims to evaluate process instability and implement stabilization strategies for improving the filling process during scale-up. Chapter two focuses on defining quality indices for the output of the filling process designed for hiPSC products. In this chapter, three quality indices are proposed, which measure the physical and biological attributes of the output. Chapter three begins with the calibration method for the filling equipment and the development of an algorithm for analyzing process instability. The algorithm detects outliers in the quality index measurements and classifies process instability as fluctuations within a batch and variations between batches. It quantifies process instability as the difference between the standard coefficient of variation (CV) and CV for stability to get CV for instability.

Chapter 2: Defining quality indices for the hiPSC filling process

This chapter defined quality indices, namely *V/V**, *D/D**, and P/P^*, for quantifying the quality attributes of the output from a filling process and observed the trends of these indices as a result of interactions between the input material and the process parameters. The quality index V/V^* measures the fill volume in each vial from a batch. D/D^* measures the particle density within each filled vial when a suspension is used as the input material. P/P^* measures the biological attributes when a cell suspension is used as the input material for the filling process. It is derived as a product of previously defined viability indices [2] The yield (*Y*) for the process was defined as the product of the above-mentioned quality indices based on the nature of the input material to understand process stability. After observing the trends of quality indices, it was determined that the mixing operation is an essential part of the filling process to maintain output homogeneity when a suspension was used as the input material. It was also determined that the mixing operation affects the fill volume of the final vials filled in a batch. The biological quality index had higher differences between samples of the same batch and saw considerable degradation after filing. Strategies such as the filling process at 4 ° C were implemented based on previous studies to mitigate the degradation of the biological attribute [3].

Chapter 3: Analysis of fluctuation within batch and variation between batches

In this chapter, an analysis method that utilizes a nonparametric, unsupervised outlier detection algorithm (Isolation Forest) to quantify stability and instability in a filling process was developed. Initially, a new method for establishing the threshold of outlier classification for quality index measurements was developed. After establishing the threshold of outlier

classification for each quality index, fluctuation and variation between batches for each of those quality indices were quantified and compared. The quantification was done with CV stability (δ) and CV for instability (δ`) derived after analysis with the algorithm. Initially, filling order-dependent fluctuations were analyzed for the three quality indices. V/V^* had order-dependent fluctuations for vials towards the end of each batch. Order-dependent fluctuations for D/D^* were only observed for the filling process without mixing operation. hIPSC suspension was always filled with mixing operation to avoid heterogeneity in the output that may affect the viability and proliferation assays. Five batches were analyzed for quantifying fluctuations within batches, and ten were analyzed for determining variation between batches. Based on this assessment, the filling process stability for different types of input materials was compared. It was shown that the filling process was more stable for the physical quality indices. If not, it could be made more stable by introducing operations like mixing during the filling process. Cell potential (P/P^*) after the filling process fluctuates more than physical quality indices even after implementing some improvement strategy.

Chapter 4: General conclusion and future perspectives

Process instability was quantified after defining quality indices for the output of the filling process and analyzing fluctuations within a batch and variation between batches using an algorithm that utilizes nonparametric statistical techniques. Furthermore, process improvement strategies were implemented based on this analysis. However, further investigations must be done to mitigate the fluctuations observed in cell potential, possibly from mechanical stress due to mixing operation. This approach can be implemented while designing a filling process for hiPSC and similar cell-based products.

Chapter 1
General introduction

1.1. Human-induced pluripotent stem cells (hiPSC) and its applications

Large-scale manufacturing of human-induced pluripotent stem cells (hiPSC) has become necessary due to their application in understanding developmental biology and diseases, creating organoids for drug screening, and being applied in cell-based therapies. Cell-based therapies utilizing somatic and stem cells are a derivative of tissue engineering, and it is a crucial component of cellular regenerative medicines[1,2]. hiPSCs potential to differentiate into specialized cells have critical implications and makes them a suitable source for regenerative medicine [3]. The prospect of cell-based therapies utilizing somatic stem cells or hiPSCs for multiple applications makes it a platform technology[4]. The manufacturing scalability of this platform technology is crucial for its widespread adoption. Cell therapy manufacturing requires intense research and development to meet the demands of potential patients. A challenge for this industry to manufacture at scale is the fundamental difference between cell-based products and conventional small-molecules, biopharmaceuticals, and biologics [3,5,6].

As stated above, hiPSC manufacturing falls under the broader umbrella of cell-based regenerative medicine. hiPSC has proven to be an excellent source for both autologous and allogenic stem cell therapy [7]. The starting material is sourced from either patients or donors, and these are subjected to a reprogramming process by ectopic expression of specific genes (e.g., Oct-4, KLF4, SOX-2 and c-Myc [OKSM]) to derive induced pluripotent stem cells [8–10]. These induced stem cells have advantages over other somatic stem cells and circumvent

some ethical issues faced by embryonic stem cells; therefore, they have a higher probability of widespread adoption [4,11]. Estimates for the number of viable cells required in a cell-based therapy are unclear, but they are likely to be around 10^9 cells per dose and require multiple doses [1,5]. The increase in demand for hiPSCs requires development in the manufacturing processes, which are fundamentally divided into upstream and downstream processes. Upstream processes focus on expanding the cell culture (increasing the number of viable cells) and downstream processes focus on the harvest, purification, formulation and cryostorage of cells derived after cell expansion [1,12–14]. The technologies implicated in the upstream processes have received extensive research and development, making the downstream unit operations a bottleneck in cell manufacturing [15,16]. Depending on the nature of the final product (either autologous or allogenic) the strategy to scale manufacturing can be split into scale-out or scale-up approaches [1,17]. A scale-out approach is suitable for autologous products as multiple batches can be prepared in parallel for various patients in a personalized manner. In autologous therapy, cells are collected from the donor, expanded in-vitro and finally administered back to the donor for treatment [17,18]. Immunotherapy is an example of autologous therapy where immune cells collected from the donor are used to cure cancer [19–21]. Allogenic cell therapy follows an off-the-shelf approach which requires scale-up of production processes in contrast to autologous cell therapy; here, donor-derived cells are reprogrammed and used to establish a master cell bank (MCB) from which cells are expanded according to requirement [22,23]. These products are more characterized than autologous products. Batch-to-batch variability arising from the inherent nature of cells is reduced since the source material remains the same for different batches [2,17,24]. The use

of hiPSCs in both autologous and allogeneic therapy is gaining momentum. hiPSC-derived hepatocytes, cardiomyocytes, and retinal progenitor cells are already at the human trial stages. Both autologous and allogenic products have their advantages and disadvantages. Autologous products are an excellent example of personalized medicines and tend to be patient-specific. The drawbacks of immune rejection is avoided in contrast to allogenic products [25–27].

Regardless of the type of cell-based therapy, both allogeneic and autologous products derived from hiPSCs require adequate production capability to meet the growing demands [28,29]. Manufacturing cells requires process development which is significantly different from conventional pharmaceuticals or biopharmaceuticals since the viable cells are the required products [5,30]. As stated previously downstream processes in cell manufacturing have become a bottleneck and require further research and development. Designing robust and scalable downstream processes is crucial to maintain the quality of the cells produced during the upstream processes, and failures at this stage are not desirable because of the substantial amount of time and resources spent [31,32].

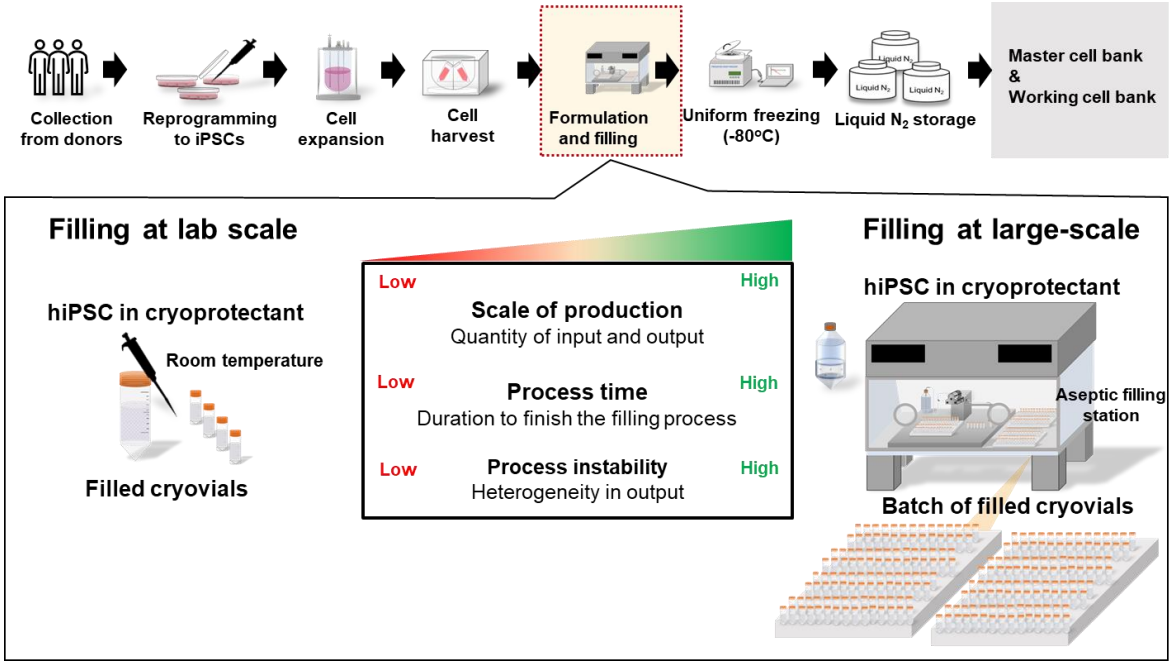


Figure 1.1 A typical hiPSC manufacturing process with the upstream and downstream unit operations. The filling process is part of downstream processes and requires understanding the process instability during scale-up. There are differences between samples (output) of the same batch and samples between batches.

1.2. Concept of cell manufacturability

Cell manufacturability focuses on designing processes specific to cell-based products, and it takes into account the complexity observed in the process output due to the transient nature of biological attributes. It tries to bridge the gap between the engineering and biological aspects of the manufacturing process [33]. The approach is different from conventional process design for pharmaceuticals, where the product attributes are much more stable and have less inherent variation. Viable cells are not in a steady state and therefore, environmental cues easily influence biological attributes. Apart from environmental cues, operator-to-operator variability and variability in the input material can also influence the

quality of the output [34,35]. The high intrinsic disorder observed in viable cells is a challenge for the reliable production of cells. In order to design cell manufacturing processes, it requires in-depth knowledge of the process parameters and product attributes [36]. The quality by design (QbD) paradigm is implemented to obtain quality products for cell therapy and design stable and capable processes [37,38]. The process design starts with understanding the input materials and defining specific quality indices for the output, which can be measured to evaluate the process performance. Once the quality indices are measured, the process can be improved according to the feedback generated from the analysis. Similar to conventional pharmaceutical or biopharmaceutical products, the process design is preceded by product design. The product design begins with defining the quality target product profile (QTPP), after which the product's expected quality attributes (QAs) are identified [39–41]. Once the QAs are defined and identified, a stable and capable process for producing the desired output is designed. Some QAs directly affecting patient health are later designated as critical quality attributes (CQAs) according to the regulatory body's requirements. The CQAs are influenced by the process parameters and amongst the process parameters, some directly impact the CQAs [42,43]. These process parameters that are crucial for achieving and maintaining the process output quality are called critical process parameters (CPPs). The interaction and combination of CPPs and QAs, along with the input material attributes for a process, define the design space (DS) for a specific product. The sequence of events from product design to process design, final validation, and production are components of all industrial manufacturing [44]. Multiple regulatory bodies are working on the standardization of these events and preparing the framework for achieving the

standards set by the regulatory body before the products are available for the customers [2,45]. The development of pharmaceutical and biopharmaceutical products can be used as a baseline for developing cell-based products and the process parameters associated with their production.

1.2.1. Fill and finish process design for cell manufacturability

Fill and finish in cell manufacturing refers to downstream processes after the cells are harvested and formulated. The cells are suspended in a cryoprotectant medium and aliquoted into vials or cryobags before they are frozen and stored [46]. The filling process for conventional pharmaceuticals or biopharmaceuticals differs from cell manufacturing because the output contains viable cells [47–49]. The sensitivity of cells towards various stress and subsequent degradation should be considered during process design. The conventional filling process is usually followed with lyophilization in the case of pharmaceuticals so that the active pharmaceutical ingredient can be stored stably [49–51]. The filling process is followed by the freezing process and cryopreservation for cell manufacturing. Since the cells need to be frozen and cryopreserved, the formulation contains cryoprotective agents like dimethyl sulfoxide (DMSO) [52]. The impact of DMSO on cell viability due to extended periods of exposure during scale-up of the filling process is documented [52–54]. Designing a filling process by considering the challenges of cell manufacturability requires defining quality indices for the output and analysis of process instability. Quality indices are indicators of QAs defined during the product development phase [52]. Depending on the nature of the quality index, assays for measurement and analysis strategies must be developed. The

characteristics of the quality index determine the appropriate analysis methods. For physical attributes which are relatively stable compared to biological attributes, conventional statistical analysis can be utilized. Analyzing the process stability and capability is crucial to determine the manufacturability of a process, and this is done with statistical process control (SPC) [55–57].

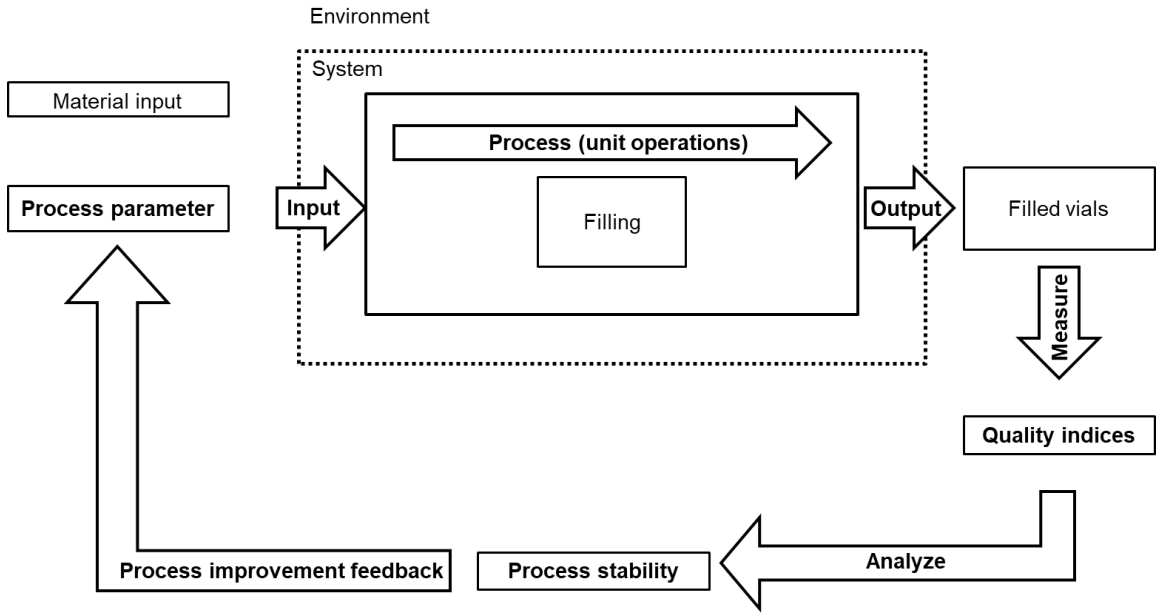


Figure 1.2 Process design scheme for filling of hiPSC considering cell manufacturability

1.3. Stability analysis for process design

SPC is utilized to analyze the data from the output measurements and conclude if the process is in statistical control. The analysis forms an important part of the process design as it generates feedback for process improvement. A process must be stable and capable for being considered manufacturable [58]. The source of possible instability should be identified, and mitigation strategies should be implemented. SPC for quality management is an

established approach implemented in various industries, and multiple tools have been developed over the years, depending on industry-specific requirements. Process design utilizes SPC to determine the process stability before process capability studies are done to improve quality requirements [59,60]. After the measurement assays, the data collected is analyzed using SPC tools. The conventional SPC tools are parametric and based on a statistical model such as the normal distribution [61]. Process instability is detected when a measurement is not within the permitted deviations of the model. This unpermitted deviation has been given multiple nomenclatures, such as outliers, faults, anomalies, or rare events [62,63].

Unlike parametric analysis, a nonparametric data-driven approach for detecting instability is robust against outlier measurements, where outliers influence the detection [64]. Modifications of the conventional tools to meet the requirements of biologics manufacturing is also reported. Control charts have been used in quality control of biologics like blood components by modifying the underlying statistical model [56,65]. Apart from the limitations of model assumptions, the control charts are also affected by the sample size, usually requiring large data samples, which may not be available during the early phase of process development. An alternative to the conventional SPC is the use of machine learning techniques to detect outliers in the output measurements [66,67]. Due to improvements in computational capacity, machine learning techniques are being utilized in multivariate analysis and advanced statistical process control. The data from the bioprocessing of stem cells is expected to have multiple attributes of interest (high dimensional data). Therefore,

techniques such as PCA, clustering, neural networks, decision trees, and random forest are considered for analysis [68].

Once the QTPP is defined, a suitable process is developed which is robust and reliable. The reliability of the process ensures product consistency and process reproducibility. A process should be designed so that the output variation is within a certain limit and can be predicted with appropriate statistical tools. The process parameters need to be optimized to achieve process control, which is done with statistical tools such as the design of experiments (DoE) [69]. The design and process optimization are continually refined with analysis feedback. For cell manufacturing, maintaining the desirable biological attributes are crucial for commercializing the product. The control strategies to achieve process reliability is complex compared to conventional pharmaceutical productions due to the challenges in cell manufacturability as previously described. Biological attributes require multivariate analysis compared to traditional manufacturing, where univariate analysis is sufficient. The unit operations within the cell manufacturing processes synergistically affect the attributes of the output. Robust analysis of attributes is necessary for process parameter optimization, which determines the batch size. The analysis must also help bridge the gap between engineering and biological aspects to attain cell manufacturability [33,70]. Process monitoring is crucial to implement QbD, which requires the development of appropriate analysis methods.

The conventional analysis methods also tend to be a univariate analysis which is not suitable for complex cell manufacturing processes involving multiple processes and their interactions. The industry standard for SPC is the control chart developed by Shewhart, which

relies on the normal distribution to detect out-of-control measurements . A process is considered to be under statistical control if the output measurements are within three standard deviations from the average [71]. This tool's reliance on the normal distribution makes it unreliable for attributes that are not in a steady-state and continually change during the process. In cell manufacturing, monitoring viability and other markers associated with biological attributes require a multivariate analysis to successfully diagnose process instability [72]. The monitoring further helps maintain the process under control and improve performance. Process design based on detection of process instability requires the development of maximally sensitive techniques and robust to all possible instabilities encountered during analysis. The analysis can be classified into three approaches: data-driven, analytical, and knowledge-based. The data-driven measures are derived directly from empirical data, while the analytical approach uses mathematical models based on first principles, and the knowledge-based approach uses causal analysis, expert systems and/or pattern recognition [62].

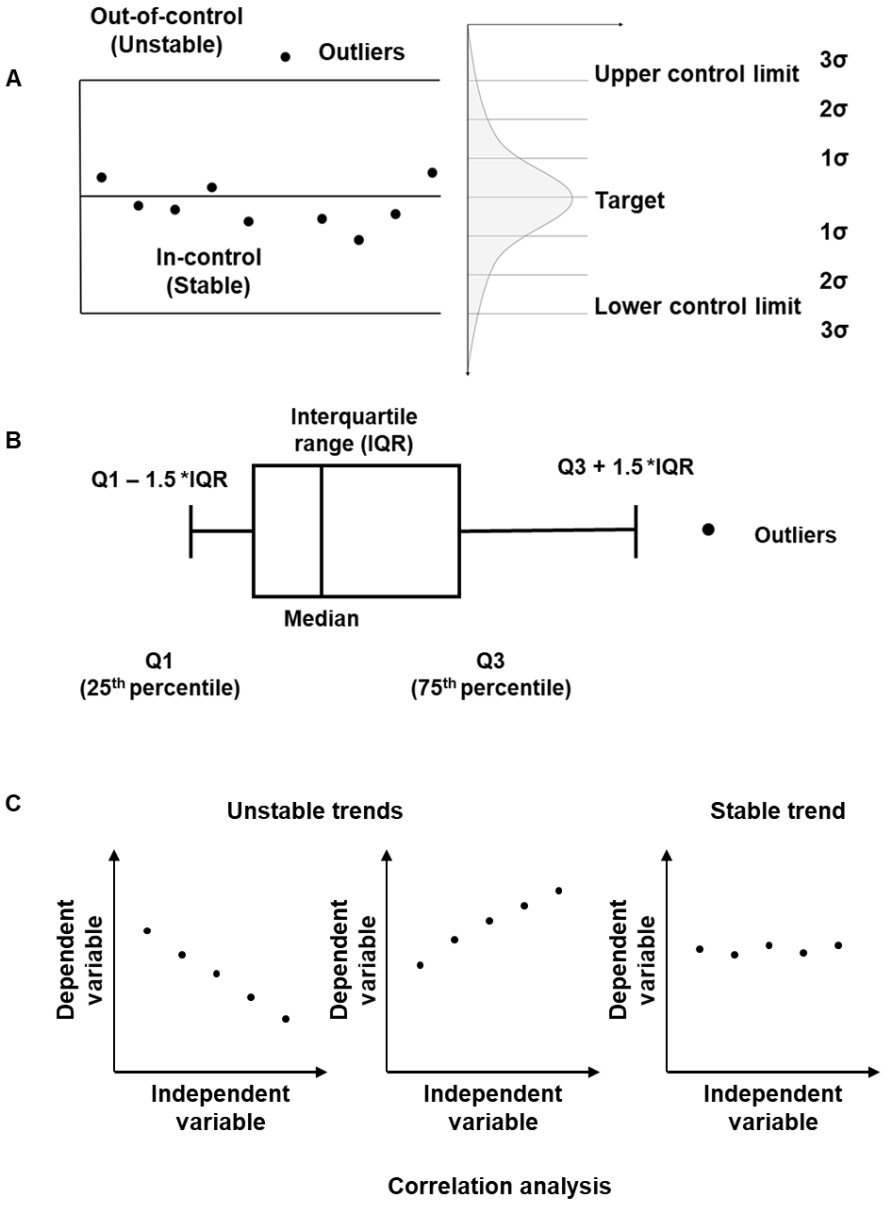


Figure 1.3 Conventional analysis for determining process stability. (A) Control chart based on parametric analysis (B) nonparametric analysis with boxplot (C) correlation analysis.

1.4. Chapter outline

Filling process design for hiPSC products considering the challenges of cell manufacturability requires evaluation of process instability. Since the analysis of biological attributes with conventional SPC is not recommended due to the limitations of parametric analysis, the current study focuses on the development of quality indices and a nonparametric analysis algorithm for the hiPSC filling process. The thesis aims to evaluate process instability and implement stabilization strategies for improving the filling process during scale-up. The study is divided into two chapters, as shown in (Fig 1.4).

Chapter two focuses on defining quality indices for the output of the filling process designed for hiPSC products. In this chapter, three quality indices are proposed, which measure the physical and biological attributes of the output. After defining the quality indices, they are measured to observe their trend.

Chapter three begins with the calibration method for the filling equipment and the development of an algorithm for analysing process instability. The algorithm detects outliers in the quality index measurements and classifies process instability as fluctuations within a batch and variations between batches. It quantifies process instability as the difference between the standard coefficient of variation (CV) and CV for stability to get CV for instability. Finally, the process instability for each quality index is categorized using the algorithm and summarized. The stabilized process is compared with the regular process to quantify the influence of stabilization strategies.

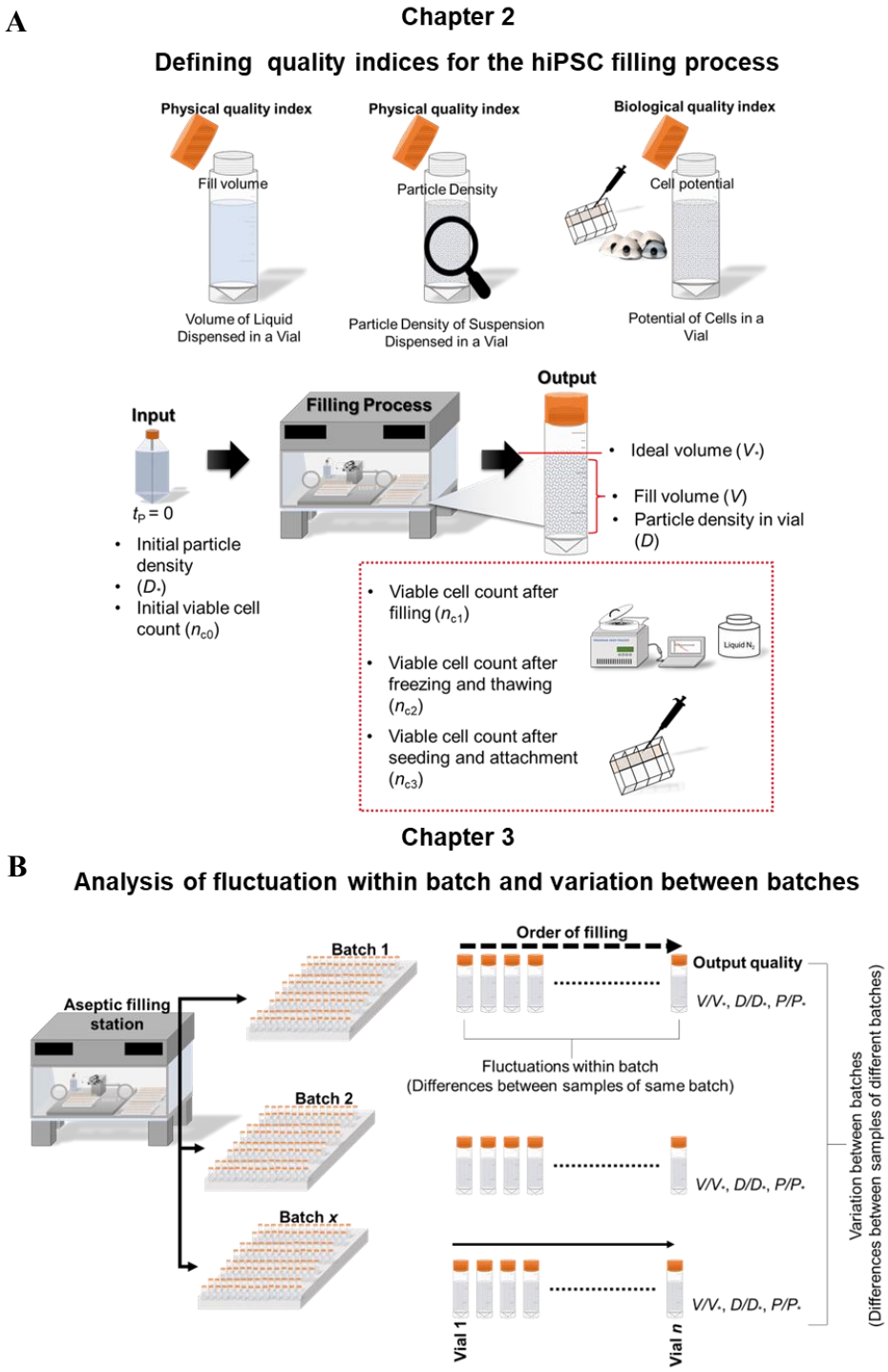


Figure 1.4 Outline of study. A) defining of quality indices for the output of the filling process. B) Analyzing fluctuations within batch and variation between batches for the filling process.

Chapter 2
Defining quality indices for the hiPSC filling process

2.1. Introduction

Identifying the quality attributes is necessary to understand the impact of the process on its output. Quality attributes are indicators of physical, chemical, biological or microbiological properties or characteristics used to assess the final quality of the output from a unit operation or the quality of the final product after a series of manufacturing processes [42]. Regulatory authorities later review these before the product is released for its intended use. As previously mentioned, identifying QAs and later CQAs is crucial to the QbD paradigm [39,73]. It is the first step in evaluating manufacturability. For these quality attributes, specific indices are defined that are measured during or after the completion of the process. The measured quality indices are analyzed to evaluate the process stability and capability from which feedback is generated to either maintain or improve the process. Conventionally once the quality indices are measured, SPC is used to analyze the process stability [59]. The variations in the output are statistically modeled, and any measurements detected outside the model's limits are treated as outliers due to process instability. If the indices are not properly defined and identified analyzing process stability and capability cannot be performed and therefore manufacturability cannot be determined. The importance of properly defining quality attributes and identifying indices to measure them cannot be overlooked. All quality analysis of the final product and stability analysis of the process depend on establishing quality indices that capture critical properties of the product and its interaction with the unit operation during the production process [39].

In this chapter, quality indices for the filling process output are defined and their trend after the filling process is analyzed. Three quality indices are proposed that measure the physical and biological attributes of the output. One of the quality indices, V/V^*, measures the volume of the input liquid filled in a vial. It is the ratio of the actual volume of a liquid filled in a vial over the target fill volume. D/D^* is the second quality index that measures the density of particles in the output. It is the ratio of particle density in a filled vial over the target particle density within the input reservoir. The final quality index P/P^* measures cell potential (viability). It is a product of three viability indices previously defined.

Physical quality indices are stable and are less challenging to define and measure, while the biological quality index is a product of three viability indices proposed by Kagihiro et al. in 2018. Quantitative measurement of the biological quality index is challenging due to the complex and highly dynamic nature of viable cells and cell preparations. The varying sensitivity of cells, the intrinsic disorder associated with them, and their interaction with process parameters of different unit operations make it difficult to define quality indices and properly measure them [74].

The trends of the quality indices are observed after the filling process, and their dependence with process time is determined. Dependence on the process time (t_P) is evaluated with nonparametric correlation analysis (Spearman's rank correlation). The chapter lays the foundation for the development instability analysis algorithm by defining the quality indices and observing their trends.

2.2. Materials and methods

2.2.1. Calibration of peristaltic pump

The peristaltic pump was calibrated before filling operation to minimise variation from poor calibration. After the reservoir was filled with input material, the position of nozzle inside the reservoir was adjusted to 5 mm distance from the bottom wall using a caliper. Once the nozzle position was fixed, the tubing was primed with the input material to reduce the peristaltic pump pulsation, and finally, vails were filled and V/V^* was measured. When five sequential vials had $V/V^* = 1$, calibration was completed.

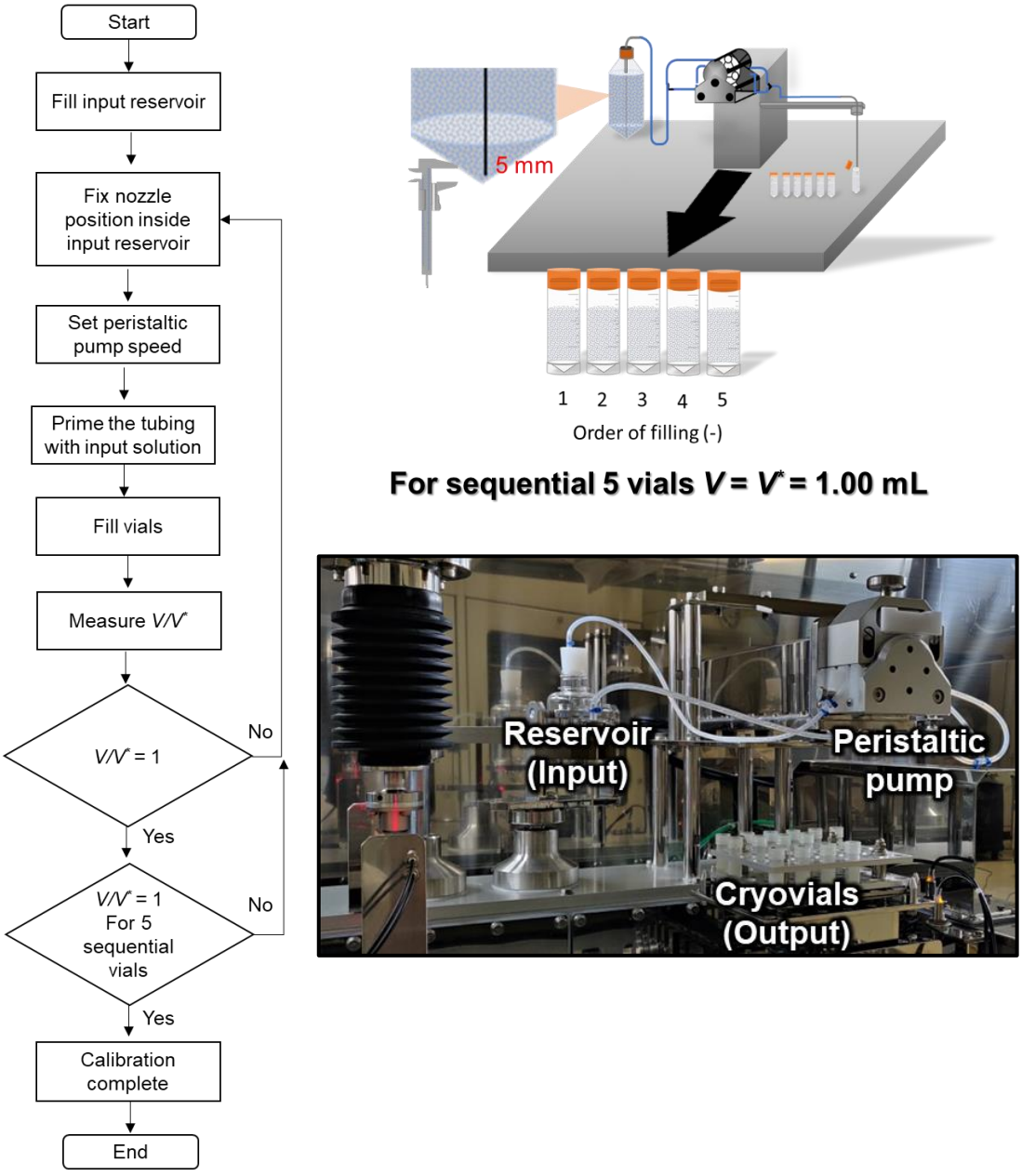


Figure 2.1 Filling process pump calibration strategy. The actual experimental setup using the filling station is shownin the bottom right corner.

2.2.2. Data acquisition for the analysis of the fill volume (V/V^*) quality index

The fill volume quality index (V/V^*) is the ratio of the amount of liquid (V) dispensed into a vial over the target fill volume (V^*). To observe the trend of V/V^* after the filling process, a commercially available cryoprotectant solution (STEM-CELLBANKER GMP grade; Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) was used. The solution was dispensed into cryovials (MS-4603WS, Sumitomo Bakelite Co., Inc., Japan) and the process was performed with and without mixing using a prototype filling station (Filling module, Shibuya Co., Ltd, Japan). The filled cryovials were weighed using a digital weighing balance (GR-200, A&D, Tokyo, Japan) and the mass of the dispensed cryoprotectant was calculated. The mass of the dispensed cryoprotectant was divided by its density to obtain the volume of liquid dispensed.

2.2.3. Data acquisition for the analysis of the particle density (D/D^*) quality index

The particle density quality index (D/D^*) is the ratio of the concentration of particles suspended in the cryoprotectant media within a filled vial over the target concentration of particles in the suspension within the input reservoir. To observe the trend for D/D^*, polystyrene beads (Copolymer Microsphere 7516A, Thermo Scientific, Fremont, CA, USA) with similar size characteristics to hiPSCs were suspended in a commercially available cryoprotectant medium at a concentration of 10^6 particles/mL. The suspension was dispensed into the cryovials while the process was performed with and without a mixing operation using the prototype filling station. The concentration of particles was calculated using an automated cell counter (TC20; Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.2.4. Culture of hiPSCs

The hiPSC line 1383D2 obtained from the Center for iPS Cell Research and Application at Kyoto University was routinely maintained by seeding at 7.5×10^3 cells/cm^2 in a commercially available medium (StemFit AK02N; Ajinomoto Co. Inc., Tokyo, Japan) in a cell culture dish with a polystyrene surface (TC-treated Culture Dishes; Corning Inc., NY, USA) coated with 0.25 μg/cm^2 recombinant laminin-511 E8 fragments (iMatrix-511; Nippi Inc., Tokyo, Japan). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and the medium was replaced every day. On day four, when the cells reached approximately 80% confluence, a subculture operation was performed. For the subculture, the cells were treated with 5 mM ethylenediaminetetraacetic acid (EDTA)/phosphate buffer saline (PBS) for 10 min at 37 °C, followed by treatment with a dissociation reagent (TrypLE Select; Thermo Fisher Scientific Inc., Waltham, MA, USA) with a 10 μM Rho-associated protein kinase (ROCK) inhibitor (CultureSure Y-27632; Fujifilm Wako Pure Chemical Industries, Osaka, Japan) for 7 min at 37 °C. Following treatment, the cells were detached by tapping or gentle flushing using a pipette, transferred to centrifuge tubes, and collected via centrifugation (180 × *g*, 3 min). Viable cells were counted by an automated cell counter using the trypan blue exclusion method and the cells were re-seeded onto new culture dishes. For the first 24 h after seeding, the culture medium was supplemented with 10 μM ROCK inhibitor [75,76].

2.2.5. Suspension of cells in a cryoprotectant medium

The cell suspension used to observe the trend of P/P^* after filling and freezing was prepared with cells harvested from 80% confluent culture dishes, as described in the maintenance protocol, and re-suspended in a commercially available cryoprotectant medium at an initial concentration of 1×10^6 cells/mL. The suspension was supplemented with 10 μM ROCK inhibitor. The addition of ROCK inhibitor to the culture medium after passage suppresses apoptotic cell death due to the stress induced from the passaging (dissociation-induced apoptosis). The concentration of 10 μM ROCK inhibitor was selected in accordance with the maintenance protocol provided by the Center for iPS Cell Research and Application at Kyoto University.

2.2.6. Freezing and thawing of cells

Cryovials filled with cell suspensions from individual batches were sampled and cryopreserved. The samples were transferred to a freezing container (Bicell; Nihon Freezer Co., Ltd., Tokyo, Japan) to achieve a uniform cooling rate and stored at −80 °C for at least 3 h. The cryovials were then transferred into a liquid nitrogen dewar (LS 3000; Taylor-Wharton, USA) and stored in vapor-phase liquid nitrogen for at least 24 h. The samples were recovered by thawing in a water bath at 37 °C for 1.5 min. Next, further viability assays were performed and the cells were seeded to analyze their proliferative potential.

2.2.7. Data acquisition for the analysis of cell potential (P/P^*)

The cell potential quality index (P/P^*) is the product of the viability indices proposed previously [52,54]. The cell suspension prepared as described previously was used to observe the trend of P/P^*. Cryovials were filled using the prototype filling station, and the cell

suspension's homogeneity in the reservoir was maintained through a mixing operation. After filling and before cryopreservation, the cryovials were assayed by an automated cell counter using the trypan blue exclusion method to calculate the survival ratio (γ). The recovery ratio (β) for the samples was calculated using the previously described viability assay after thawing and recovery. The attachment efficiency (α) of the cells was calculated as the ratio of the number of cells remaining after seeding and incubation for 24 h at a seeding density of 2.5×10^3 cells/cm^2 to check the proliferative capacity.

$$\gamma = \frac{n_{c1}}{nc0}, \beta = \frac{n_{c2}}{n_{c1}}, \alpha = \frac{n_{c3}}{n_{c,2}} \quad (1)$$

Here, n_{c0} is the viable cell count at $t_P = 0$; $n_{c1}$is the viable cell count after filling at t_P; n_{c2} is the viable cell count after filling, freezing, and thawing; and n_{c3} is the total number of viable cells on the culture plate counted using phase-contrast microscopic imaging at 40 times magnification.

$$P/P^* = \alpha \times \beta \times \gamma \quad (2)$$

2.2.8. Statistical analysis

Spearman's rank correlation

Spearman's rank correlation coefficient or Spearman's ρ is a nonparametric measure of rank correlation. The Spearman correlation between two variables is equal to the Pearson correlation between the rank values of the two variables [77]. Spearman's correlation assesses monotonic relationships and is suitable for robust correlation analysis in the presence of outlier data. Following the procedure described by Bonett et al. (2000), 20 data points were included in our analysis.

$$\rho = 1 - \frac{6\sum {d_i}^2}{n(n^2-1)} \quad (3)$$

ρ = Spearman's rank correlation coefficient

d_i = difference between the two ranks of each observation

n = number of observations

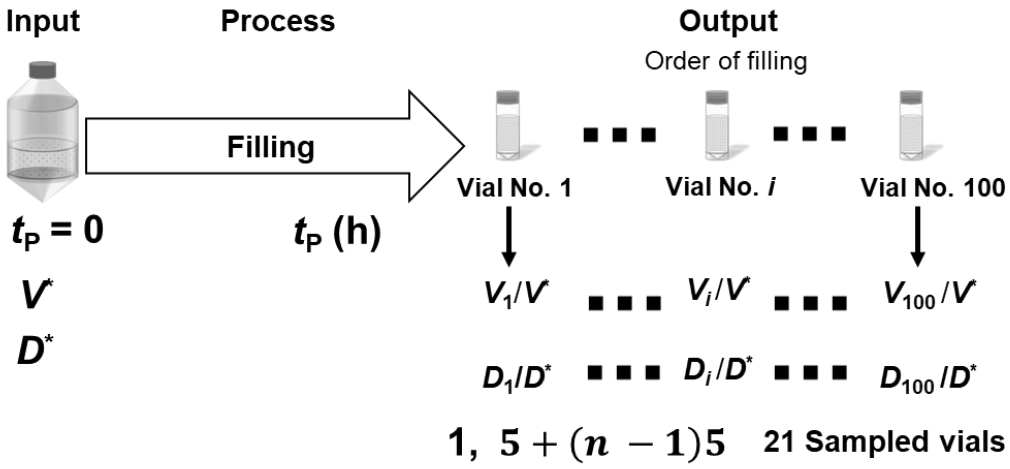


Figure 2.2 Methodology for trend analysis of quality indices V/V^* and D/D^* after the filling process. The filling is done with materials that are independent of degradation with process time.

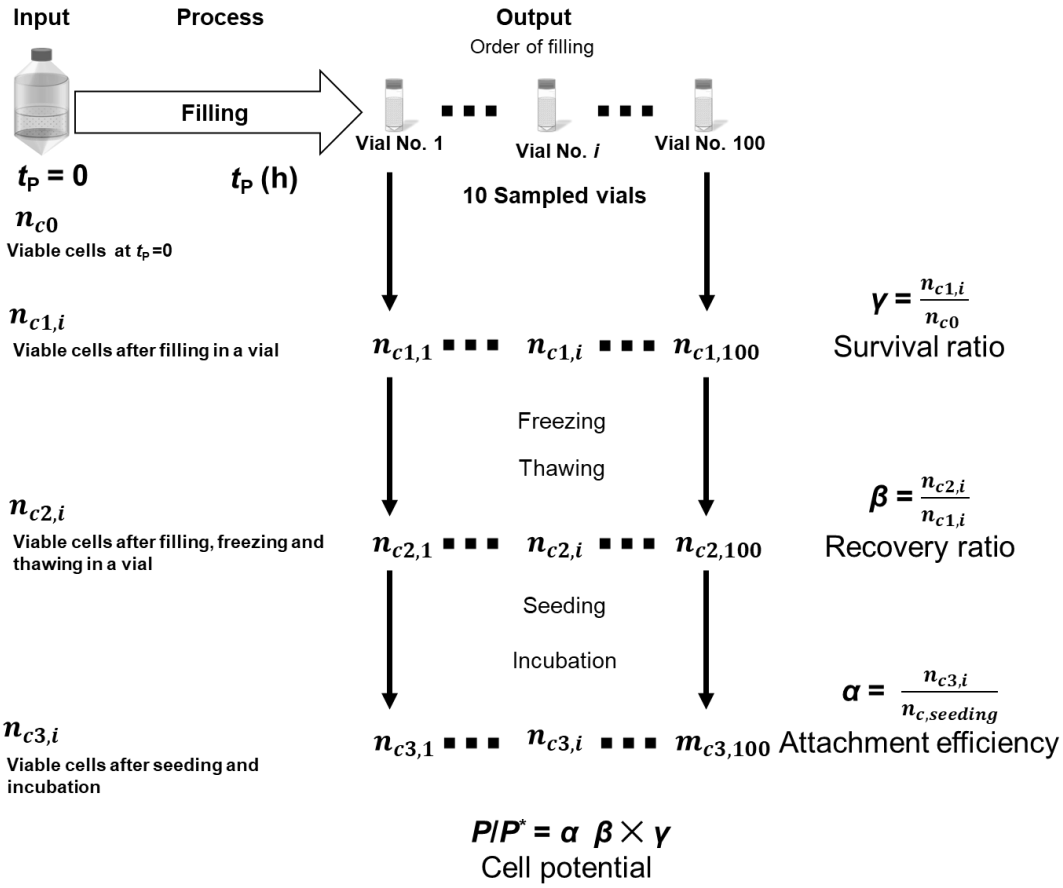


Figure 2.3 Methodology for trend analysis of quality index P/P^* after the filling process. The filling is done with hiPSC cell suspension dependent of degradation with process time.

2.3. Results

2.3.1. Trends of quality indices in a batch after the filling process

V/V_* was evaluated after the filling process was performed with and without mixing. A single batch consisted of 100 filled cryovials. Twenty-one samples (Vial No. = 1, 5*i*; $\{i \mid 1 \leq i \leq 20, i \in \mathbb{N}\}$) from each batch were measured and analyzed, and it was observed that the final samples of filled vials (Vial No. 100) for both conditions (with and without mixing) had lower fill volumes than the rest of the samples. The trends of V/V^* in the batches are presented in Fig. 2.3A. D/D^* was evaluated after the filling process with and without mixing for a suspension of polystyrene particles in the cryoprotectant medium. A batch consisted of 100 filled cryovials. Twenty-one samples (Vial No. = 1, 5*i*; $\{i \mid 1 \leq i \leq 20, i \in \mathbb{N}\}$) from each batch were measured, and it was observed that the majority of samples after filling without mixing were different from each other and the target particle density. For the filling process with the mixing operation, these differences between samples reduced and were close to the set target value (density in the reservoir). The trends of D/D^* in the batches are presented in Fig. 2.3B. P/P^* was calculated after measuring viability indices γ, β, α, and quantifying their product after the filling process with mixing for a suspension of hiPSCs in the cryoprotectant medium. A batch consisted of 100 filled cryovials. Ten samples (Vial No. = 1, 10*i*; $\{i \mid 1 \leq i \leq 9, i \in \mathbb{N}\}$) from each batch were measured and analyzed. The trend for P/P^* was observed and determined to be less than unity (suggesting a decrease in cell potentail). Additionally, It was observed that the survival ratio(γ) decreased after the filling process was completed. The recovery ratio (β) between samples was not consistent, but the overall trend was stable and close to unity. Attachment efficiency (α) between samples were similar and

above unity as the cells had multiplied after 24 h of incubation. The filling process was performed with the mixing operation because it was previously observed that mixing is necessary to maintain the homogeneity of the suspension in the filled cryovials. The trends of P/P^* and viability indices in a batch are presented in Fig. 2.3C and Fig.2.4, respectively.

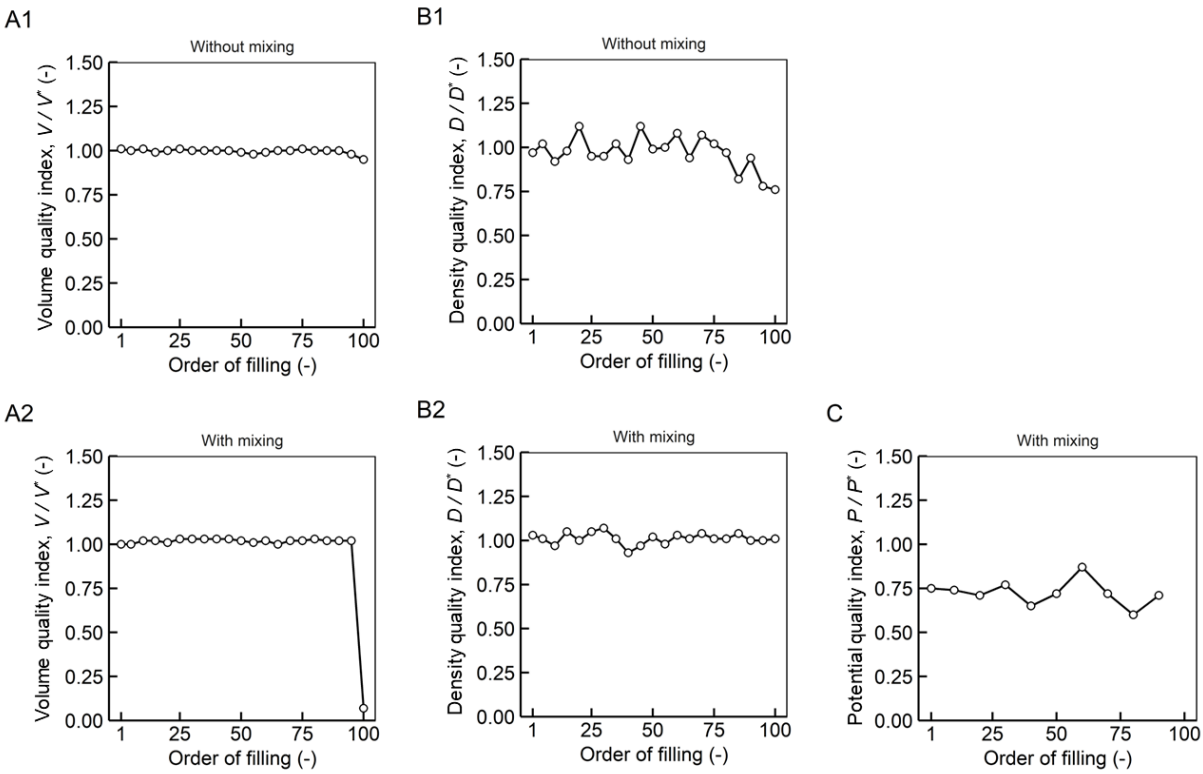


Figure 2.4 Trends of quality indices V/V^* (A1-A2) and D/D^* (B1-B2) after the filling process. Twenty-one samples from a batch of 100 filled vials are represented. The filling process was performed under two conditions with and without mixing. Closed black circles represent outlier data points and open circles represent normal data points.

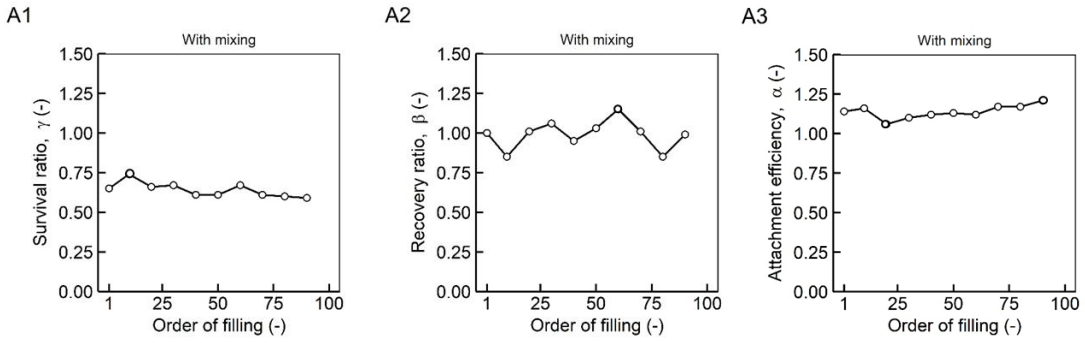


Figure 2.5 Trends of viability indices (A1 to A3) γ, β and α after the filling process at 25° C. Ten samples from a batch of 100 filled vials are represented. The filling process was performed with the mixing operation.

2.3.2. Process-time-dependence of the quality indices

The influence of Process time (t_P) on the quality indices was elucidated after the outputs of the filling process were measured for different t_P values (0, 1, 2, and 4 h). V/V^* was concluded to have fluctuations independent of t_P, as shown in Fig. 2.5A. D/D_* was concluded to have fluctuations dependent on t_P, where a strong statistically negative correlation was observed for the filling process without the mixing operation. This trend for D/D^* was not observed during the filling process with the mixing operation, as shown in Fig. 2.5B. P/P^* had a negative correlation with t_P, as did the viability indices γ and α, as shown in Fig. 2.5C and Fig.2.6, respectively.

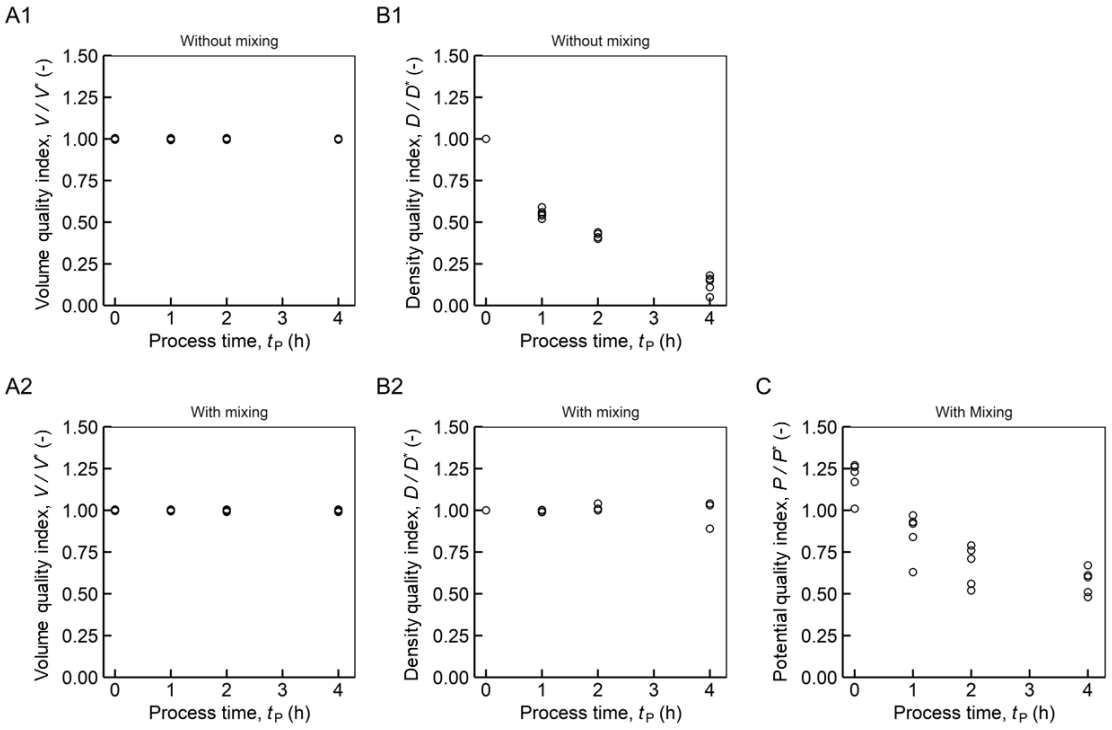


Figure 2.6 Trends of quality indices (A-B) V/V^*, D/D^* and (C) P/P^* after the filling process at different t_p. Five samples at each time point was analyzed

2.4. Trend of the biological quality index at 4º C

The trend of P/P_* was evaluated for a filling process with mixing operation at 4º C. The low temperature was selected to observe if it could maintain the biological quality index close to unity during the filling process.. Fig.2.6 represents the trend of P/P_* and viability indices. The difference between samples within the batch was comparable to the filling process at room temperature, but the quality index was maintained close to unity throughout the filling process for all the samples that were measured. The viability indices also showed a similar trend. The only viability index with considerable differences between samples was the recovery ratio (β).

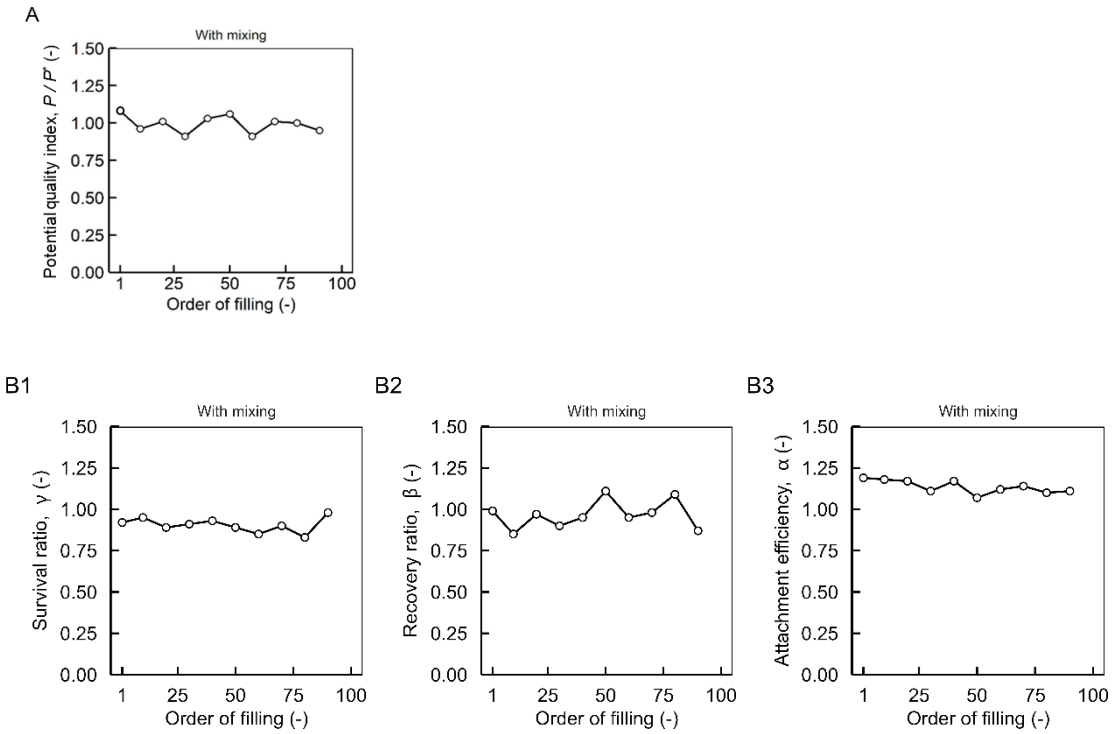


Figure 2.7 Trends of quality index (A) P/P^* and viability indices (B) after the filling process with mixing at 4° C.

2.5. Discussion

In order to understand the effects of a process on its output, it is crucial to measure the output's quality attributes. Quality attributes as previously stated can be physical, chemical, biological or microbiological characteristics/aspects of a product [2,45]. The various unit operations in the filling process interact differently with the different types of quality attributes and therefore, the impact of these interactions must be quantified for a better understanding of the overall process. Quality indices defined in this chapter are used to quantify the quality attributes of the output from the filling process. These include two physical and one biological quality indices, namely V/V^*, D/D^*, and P/P^*.

Fill volume (V/V^*) is a measurement of the amount of material (solution or suspension) dispensed to a vial during the filling process. Since it quantifies a physical quality attribute, it is not impacted by the duration of process time (independent of degradation with process time t_P). The results suggest that there is a particular trend in the differences observed between samples. The final vials to be filled within a batch (95-100) have significantly lower fill volume than the rest of the samples. The trend becomes more apparent for the filling process with the mixing operation, suggesting a causal link between lower volumes observed for the final vials and the mixing operation. This heterogeneity of V/V^* between the samples of the same batch could be due to the formation of air bubbles within the input reservoir that disrupts the target fill volume to be achieved. The use of a peristaltic pump could also contribute to this phenomenon due to pulsation that introduces air bubbles within the tubing [78,79]. The process time independence of the differences between samples was verified using correlation analysis. This was expected as fill volume, a physical quality attribute, does not degrade with an increase in process time.

Particle density (D/D^*) quality index quantifies the number of particles in a unit volume of the suspension. The quality index is relevant to understanding how the filling process affects a solid liquid (two-phase suspension). Ideally, after the filling process the particle density within each sample in a batch and the particle density within the input reservoir should be similar. However, this was not observed after D/D^* was measured after the filling process without mixing operation, suggesting that the suspension was heterogenous within the input reservoir. From the results, it can be observed that there is a considerable difference for D/D^* between samples in a batch, and as the filling progresses, the final vials tend to have

lower particle density compared to other samples. As previously stated, this heterogeneity is likely due to the heterogeneity of suspension within the input reservoir from sedimentation of the suspended particles as process time progresses. This phenomenon was confirmed as there was a negative correlation between process time and D/D^*. A mixing operation was combined with the filling process to maintain homogeneity and to confirm further that the heterogeneity was due to the sedimentation of suspended particles in the reservoir. The trend of D/D^* changed, and it could be observed that the differences between samples reduced compared to the filling process without mixing. The reduction of particle density in the final vials of a batch was also not observed (confirming the homogeneity of the suspension). The negative correlation with process time was also not observed for the filling process with mixing. These observations suggest the importance of maintaining the homogeneity of suspension within the input reservoir using a mixing operation.

Cell potential (P/P^*) quality index is used to quantify the biological quality attribute of the output from the filling process related to a cell suspension. The cell suspension is also solid in liquid (two-phase suspension) that will show similar trends to that of D/D^* if homogeneity is not maintained within the reservoir; hence the filling process for cell suspension was always performed with combined mixing operation [46,47]. The additional complexity is due to the biological nature of the suspended cells (their viability). P/P^* is a product of the viability indices defined by Kagihiro et al. in 2018. These indices, namely survival ratio (γ), recovery ratio (β) and attachment efficiency (α) measure the viability and proliferative capacity of the cells after the filling process. Even after maintaining the homogeneity of the cell suspension, differences can be observed between samples of the

same batch, and the overall P/P^* is much lower than unity, which suggests a degradation in cell potential after the filing process. The cells are complex dynamic systems that interact continuously with the external environment and with each other [35]. This clear decrease in P/P^* could be attributed to the suspended losing viability and proliferative capacity from exposure to certain cryoprotective compounds like dimethyl sulfoxide (DMSO) in the suspension media [54]. These chemicals are necessary to protect the cells from the impact of freezing/cryostorage that follows the filling process. It has been well documented that the same chemicals decrease cell viability by affecting the membrane integrity of mitochondria within cells and activating apoptotic pathways that lead to cell death [54,80,81]. The viability index γ clearly decreased for all the samples by the end of filling process and shows considerable differences between samples of the same batch. This could be due to the exposure of cells to the DMSO as well as the impact of shear stress from the mixing operation. Viability indices β and α are also show considerable differences between samples of the same batch but are still maintained close to unity. The impact of exposure to DMSO on cell potential was further confirmed with correlation analysis between process time and P/P^*. These results are in agreement with the findings of Kagihiro et al. and therefore, we followed similar mitigation steps to improve P/P^*. The improvement strategies include performing the filling process at a low temperature (4^o C) or supplementing the cell suspension with antioxidants. The former approach was implemented in our filling process and it maintained the cell potential close to unity throughout the filling process. However, the differences between samples within the same batch were similar to the normal filling process.

The quality indices defined in this chapter quantify the yield from the filling process based on the input solution/suspension characteristics. The yield can be calculated as the product of these individual quality indices.

2.6. Chapter summary

This chapter defined quality indices, namely V/V^*, D/D^*, and P/P^*, for quantifying the quality attributes of the output from a filling process and observed the trends of these indices as a result of interactions between the input material and the process parameters. It was observed that the mixing operation is an essential part of the filling process to maintain output homogeneity, but it also affects the fill volume of the final vials to be filled in a batch. The biological quality index had higher differences between samples of the same batch and saw considerable degradation after filing. Further, the yield of the filling process was established as the product of the given quality indices and this lays the foundation of further understanding the process stability.

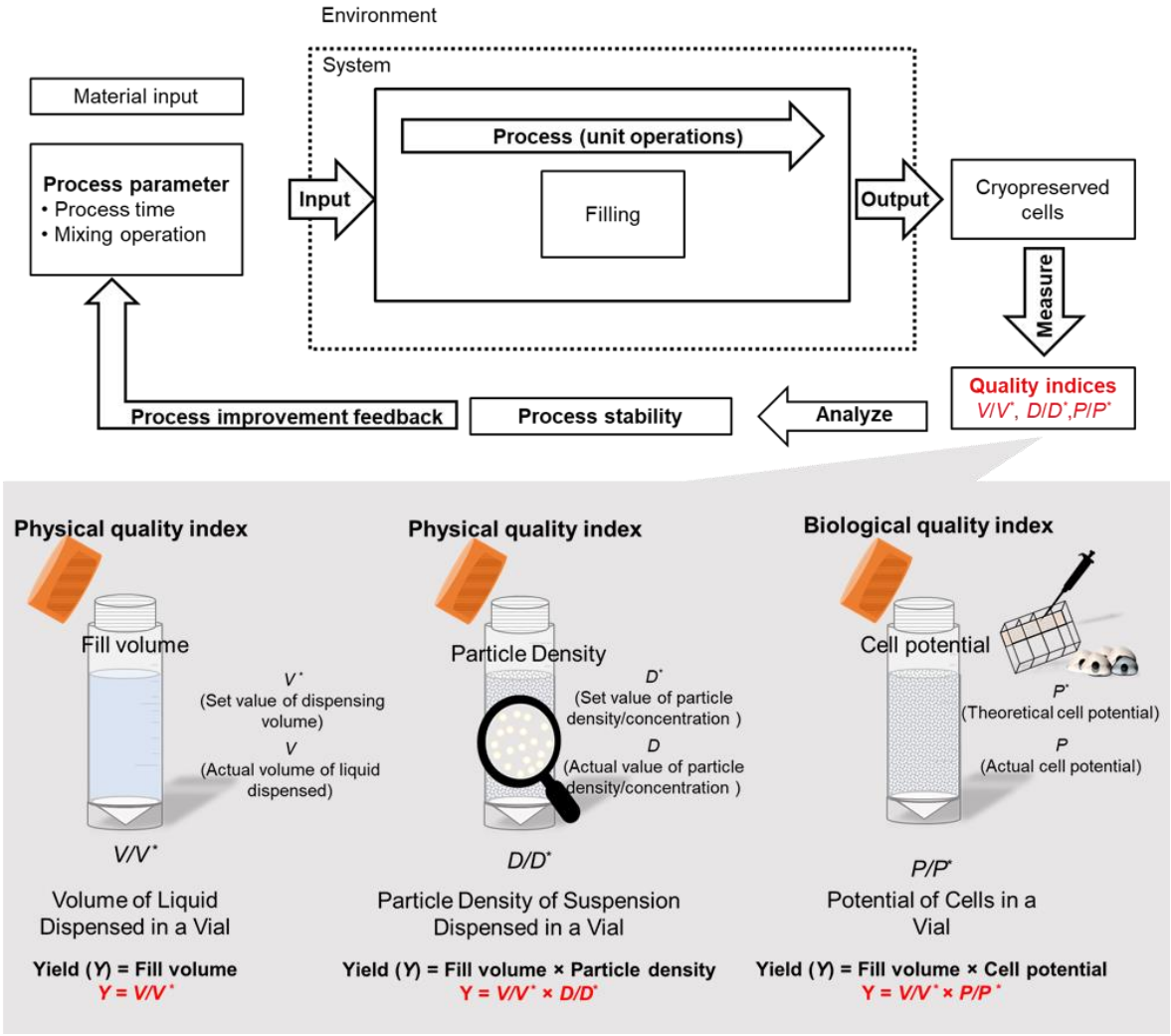


Figure 2.8 Relevance of quality indices in designing a filling process. Yield of the filling process is calculated as the product of individual quality indices corresponding to characteristics of the input.

Chapter 3

Analysis of fluctuation within batch and variation between batches

3.1.Introduction

Once the quality indices are defined and measured to quantify the quality attributes, their trends after the filling process can be observed. These trends within a batch and between batches need to be evaluated in order to quantify process stability. In the last chapter, differences between samples were mentioned, but they were not used to quantify the interactions between the unit operations and the input material. There remains a gap in understanding these trends and associating them with process stability analysis. Conventionally, SPC was used to connect the differences between the measurements of quality indices to that of process stability. This was done by detecting outlier measurements using parametric statistical models (normal/gaussian distribution) [58,59,71]. This approach is widely used in different industries but faces certain challenges when applied to analyze biological quality indices that measure attributes in a dynamic state. Robust statistical techniques are required for detecting true outliers from normal measurements. Apart from the complexity of the inherent nature of biological quality attributes, data from cell manufacturing processes tend to be high dimensional [61,72]. Therefore, a robust nonparametric statistical tool is required to process high-dimensional data. An algorithm that utilizes Isolation Forest (a amchine learning tool) was developed to detect the outliers in the quality index measurements [82,83]. The technique is based on decision trees and, since

computational capacity has increased significantly over the years, is being used more and more frequently in different industries [84].

The chapter begins with a calibration strategy for the filling process to avoid/minimize variations in the quality indices from faulty pump calibration. Once the calibration is completed, the filling process was performed with different input materials and their respective quality indices were measured. This data was used to establish a threshold for the anomaly score generated by the Isolation Forest to categorize measurements as outliers or normal. Detecting outliers is a crucial part of evaluating process instability, as this is used to establish two new indices, namely the stability (δ) and instability (δ`) index. These indices were used to quantify the fluctuation of quality indices within a batch, following which, variations between batches were also quantified.

3.2. Process stability and instability analysis

3.2.1. Isolation Forest outlier detection

The Isolation Forest outlier detection technique (Liu et al., 2008, 2012) is an unsupervised machine learning algorithm used to detect outliers in a dataset [82]. Isolation Forest explicitly identifies anomalies instead of profiling normal data points. This algorithm constructs an ensemble of Isolation Trees (*i*Trees), which are proper binary search trees (BSTs), to detect outlier data. This method isolates outliers close to the root of the *i*Tree because the average path length ($E(h(x))$) of outliers in an ensemble of trees is significantly shorter than that of normal data points. The average path length of a data point from an Isolation Forest is used to generate an anomaly score to distinguish outliers from normal data.

In the first stage of the Isolation Forest outlier detection algorithm, a subsample ψ from a dataset of n instances is selected and used to create an ensemble of *i*Trees through the random recursive partitioning of ψ. The tree height limit l is set automatically according to the subsampling size. The number of trees in an Isolation Forest is set at the beginning along with ψ.

$$l = ceiling(\log_2 \psi) \tag{4}$$

In the second stage, all data points in n are passed through the *i*Trees created in the first stage. In the third stage, an outlier score is generated for all data points based on the path length $h(x)$ of the data points in an *i*Tree. The average path length $E(h(x))$ for a data point is an ensemble of *i*Trees is divided by the average $h(x)$ for the external node termination of an unsuccessful search in a BST. Given a dataset of ψ instances, the average path length of an unsuccessful search in a BST is given by $c(\psi)$.

$$c(\psi) = 2H(\psi - 1) - (2\frac{(\psi-1)}{\psi}) \quad (5)$$

Here, $H(i)$ is the harmonic number, which can be estimated using $\ln(i) + 0.5772156649$ (Euler's constant). Because $c(\psi)$ is the average of $h(x)$ given ψ instances, $h(x)$ is normalized through division by $c(\psi)$. The outlier score s of an instance x is defined as follows:

$$s(x, \psi) = 2^{-\frac{E(h(x))}{c(\psi)}} \quad (6)$$

General conditions:

when $E(h(x)) \rightarrow c(\varphi)$, $s(x, \varphi) \rightarrow 0.5$

when $E(h(x)) \rightarrow (\varphi\text{-}1)$, $s(x, \varphi) \rightarrow 0.0$

when $E(h(x)) \rightarrow 0$, $s(x, \varphi) \rightarrow 1.0$

a. If instances return s very close to one, then they are definitely outliers.
b. If instances return s much smaller than 0.5, then they are considered as normal instances.
c. If all instances return $s \approx 0.5$, then the entire sample does not contain any distinct outliers.

In this study, 100 *i*Trees were constructed for analysis and the sample size for *i*Tree construction was equivalent to the size of the actual dataset. A data point with an anomaly score greater than established thershold was considered as an outlier.

Statistical software

Analysis was performed using R version 4.1.0 (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria). The “isofor” package for Isolation Forest outlier detection was used for analysis (Eric Graves (2021). isofor: Isolation Forest Anomaly Detection. R package version 1.0.0).

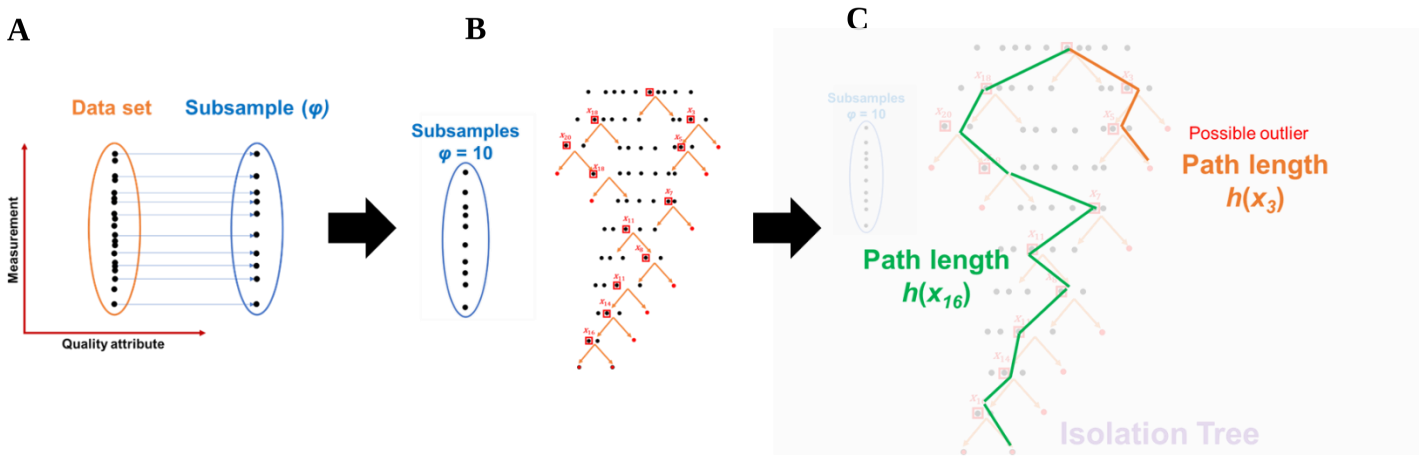
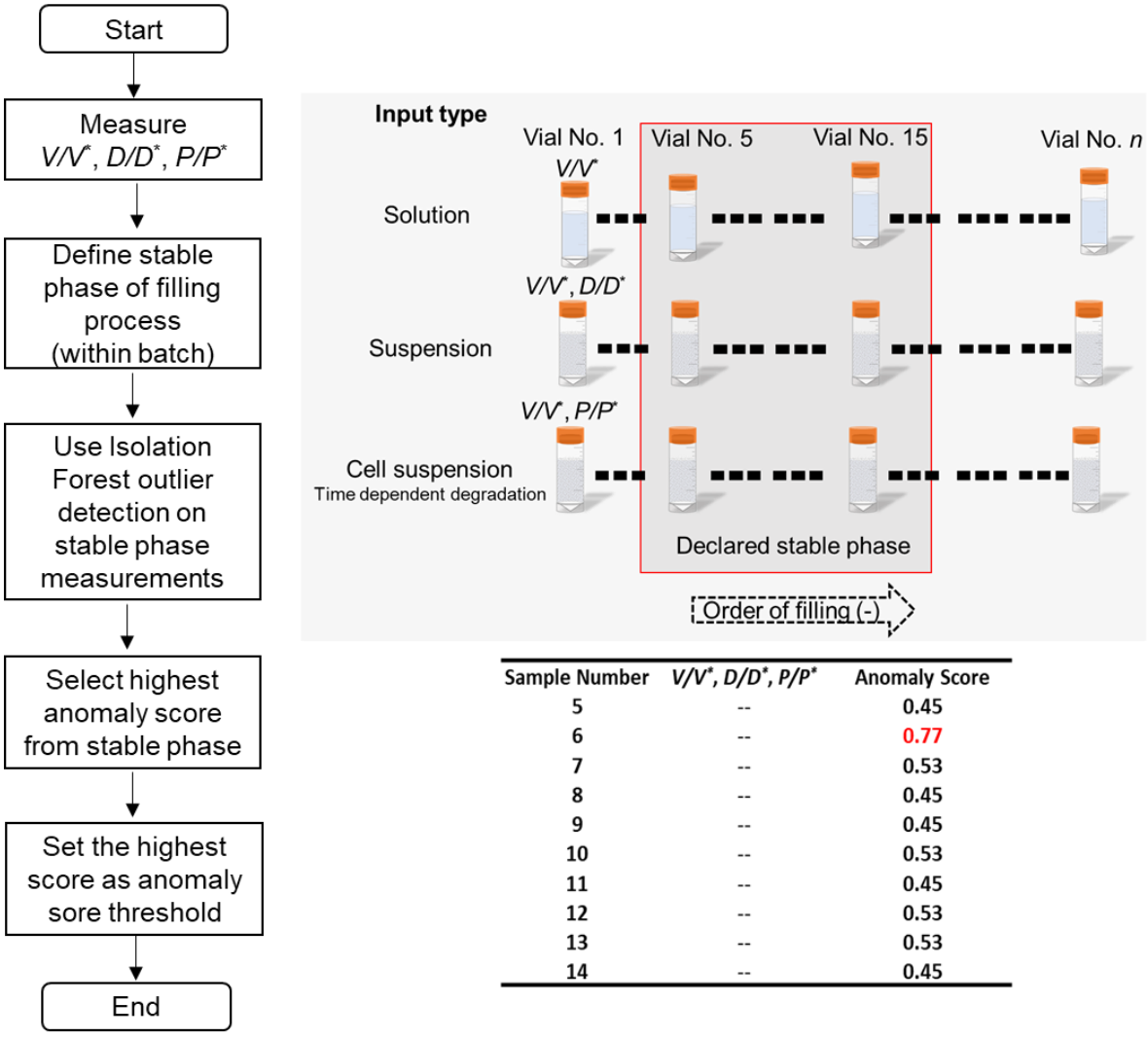


Figure 3.1 Principle of outlier detection with Isolation Forest. A) subsample is derived from the actual data set B) subsmaple is recursively partitioned to separate each data point C) partition length is used to determine if a point is normal or outler

3.2.2. Establishment of the anomaly score threshold

The threshold for anomaly scores to classify outlier measurements was obtained after quality indices from a stable phase within a batch were analyzed with Isolation Forest. The highest score within the generated anomaly score was selected as the threshold for outlier classification.



Sample Number	V/V*, D/D*, P/P*	Anomaly Score
5	--	0.45
6	--	0.77
7	--	0.53
8	--	0.45
9	--	0.45
10	--	0.53
11	--	0.45
12	--	0.53
13	--	0.53
14	--	0.45

Figure 3.2 Strategy for establishing anomaly score threshold for outlier classification.

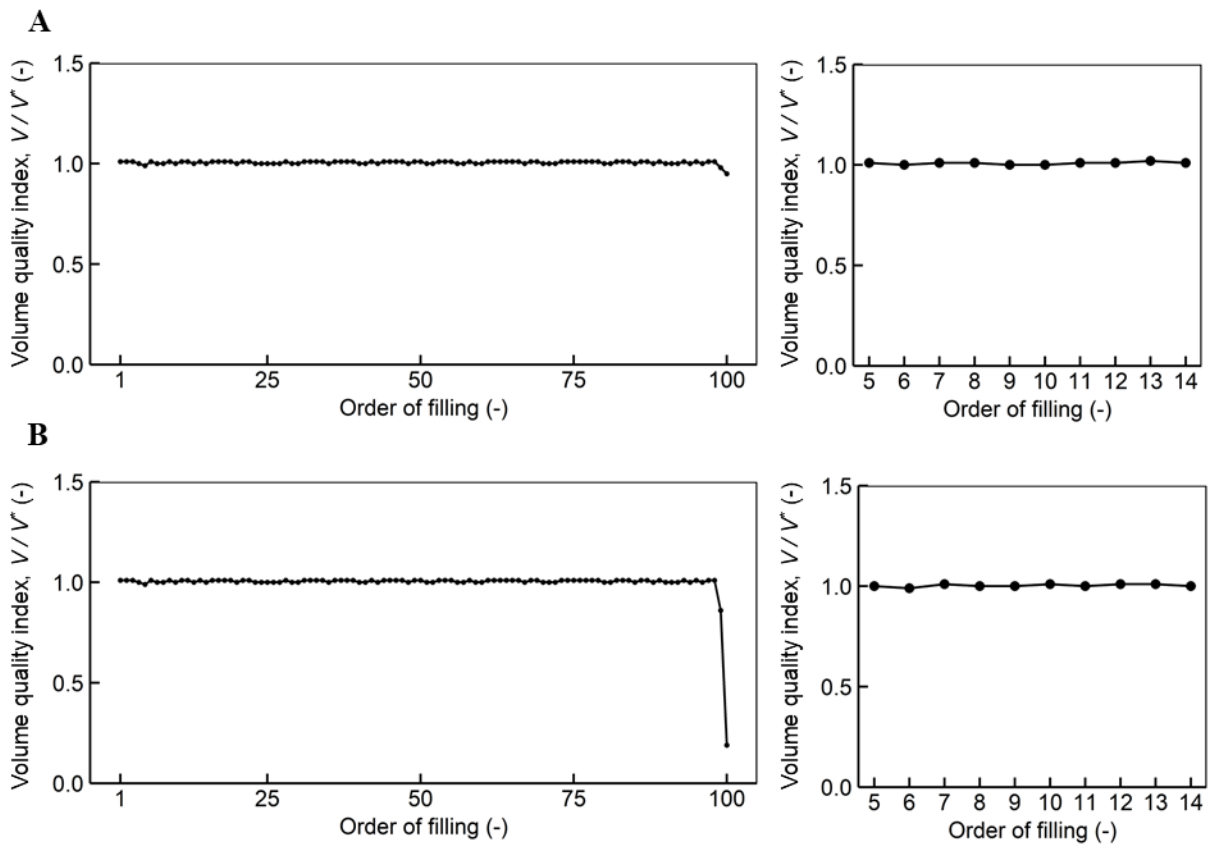


Figure 3.3 Selection of anomaly score threshold value for outlier detection. The threshold for physical quality index V/V^* A) without mixing and B) with mixing is depicted.

As described previously, the protocol for selecting the anomaly score threshold is followed to obtain it for the physical quality index V/V^*. After analyzing the anomaly score of the stable phase, 0.77 and 0.75 were set as the threshold for detecting outliers in V/V^* measurements after filling process without and with mixing respectively.

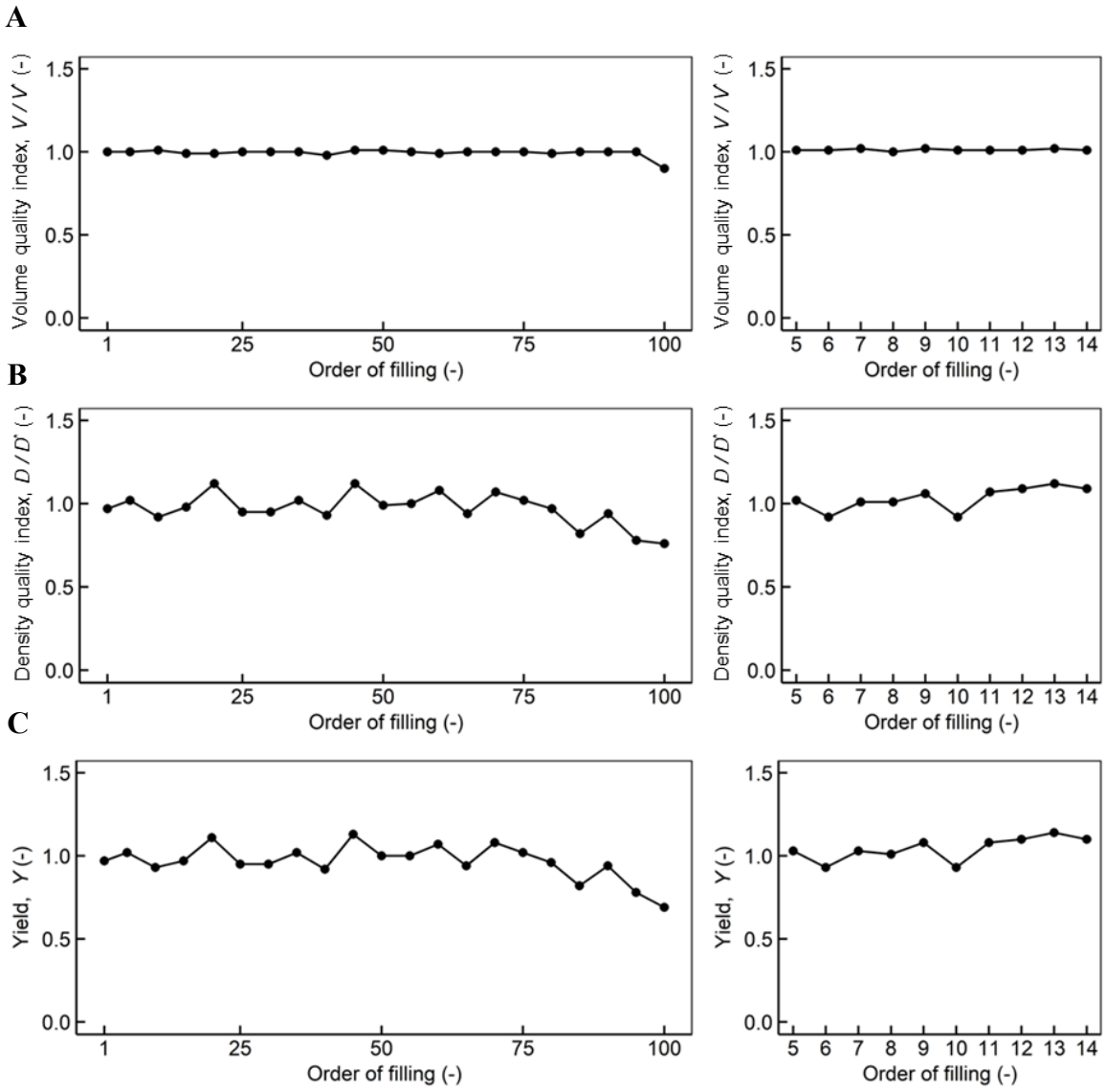


Figure 3.4 Selection of anomaly score threshold value for quality indices from a suspension. The threshold of physical quality index A) V/V^* B) D/D^* C) and yield (Y) for filling without mixing.

The protocol for selecting anomaly score threshold as described previously is followed to obtain the threshold for the physical quality indices from a suspension V/V^*, D/D^* and their yield. After analyzing the anomaly score of the stable phase, 0.77, 0.60 and 0.62 were set as the threshold for detecting outliers in V/V^*, D/D^* and yield measurements after filling process without mixing, respectively.

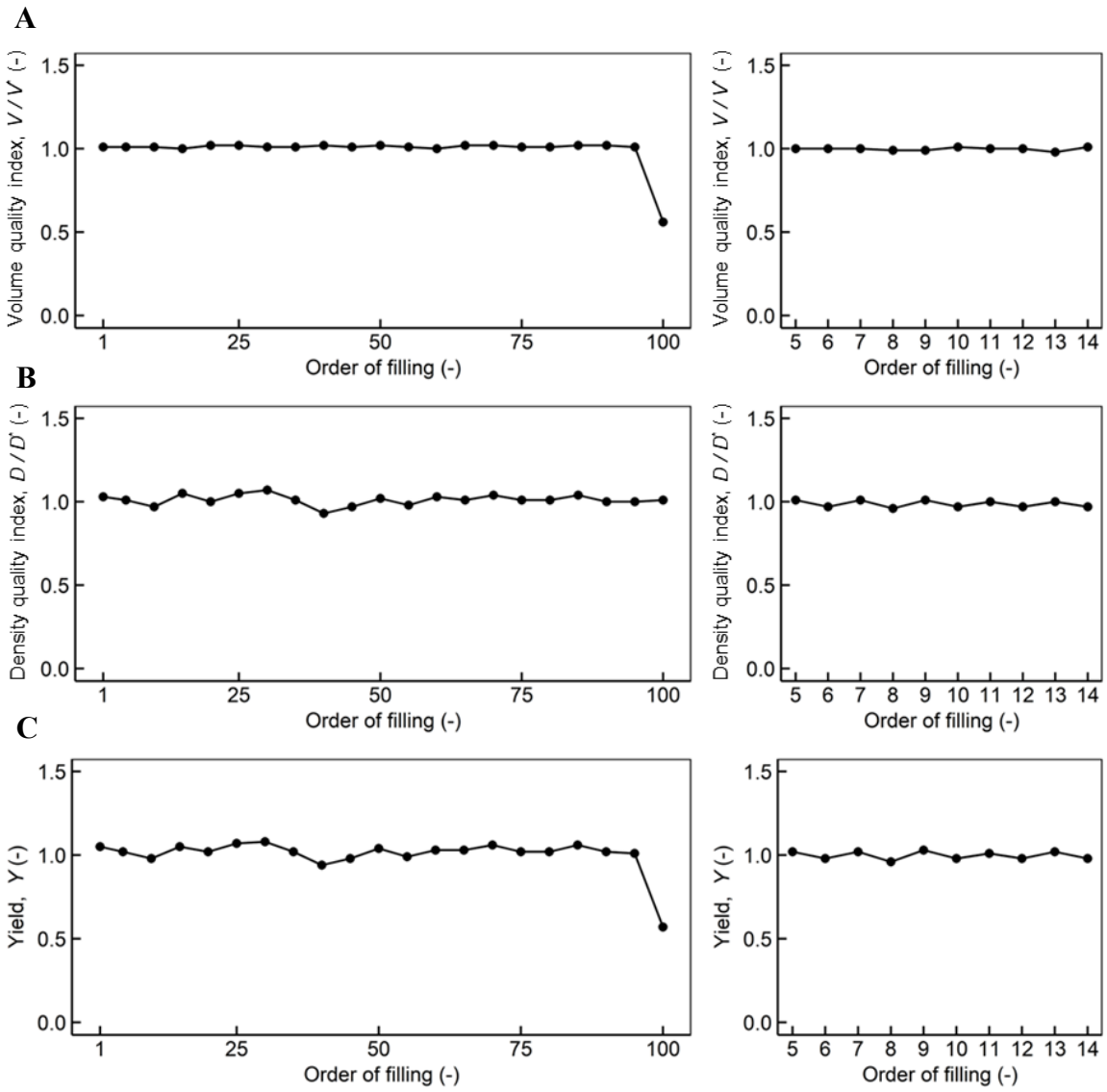


Figure 3.5 Selection of anomaly score threshold value for quality indices from a suspension. The threshold of physical quality index A) V/V^* B) D/D^* C) and yield (Y) for filling with mixing.

The protocol for selecting anomaly score threshold as described previously is followed to obtain the threshold for the physical quality indices from a suspension V/V^*, D/D^* and their yield. After analyzing the anomaly score of the stable phase, 0.72, 0.69 and 0.70 were set as the threshold for detecting outliers in V/V^*, D/D^* and yield measurements after filling process with mixing, respectively.

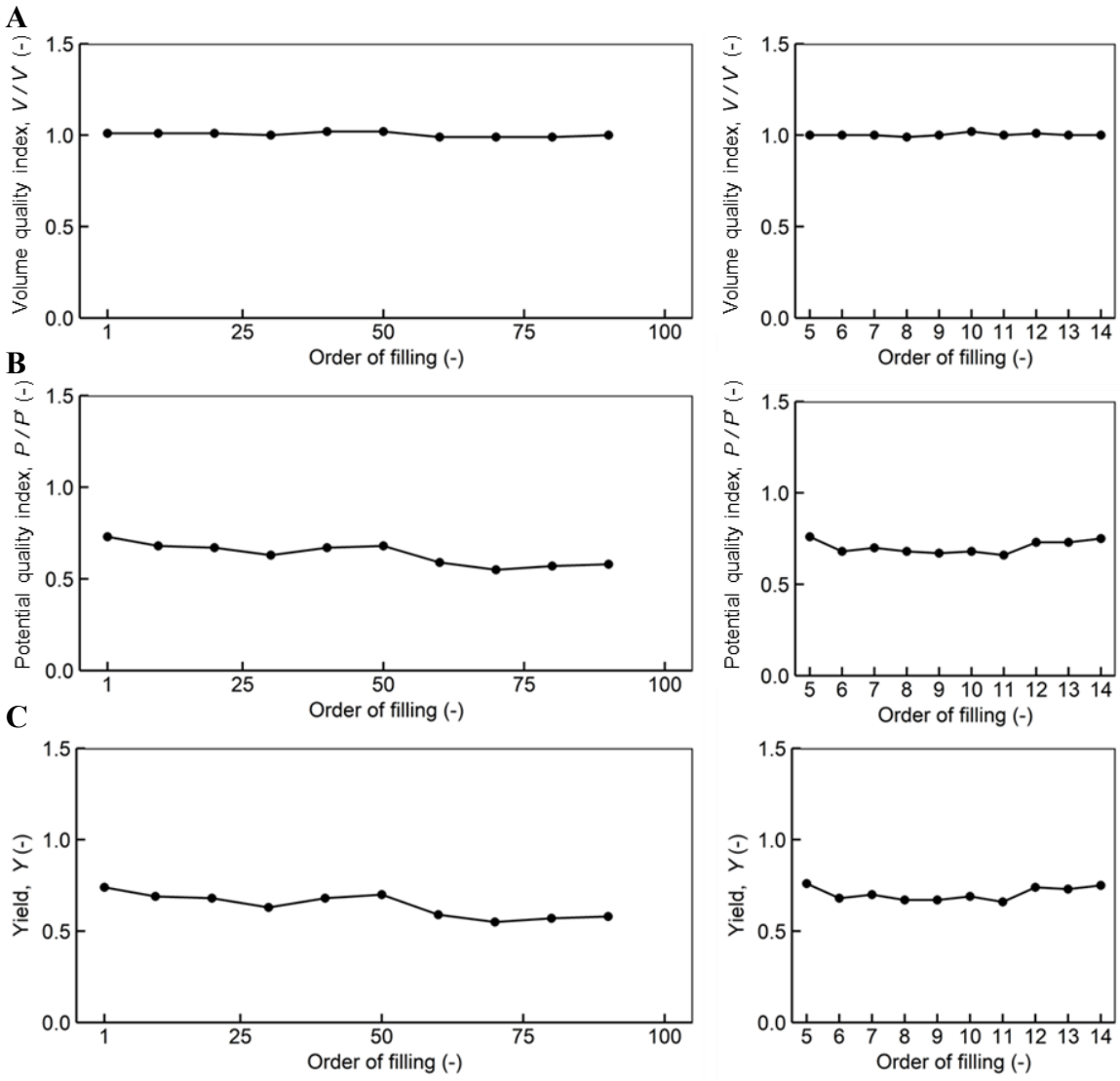


Figure 3.6 Selection of anomaly score threshold value for quality indices from a cell suspension. The threshold of physical quality index A) V/V^* B) P/P^* C) and yield (Y) for filling with mixing.

The protocol for selecting anomaly score threshold as described previously is followed to obtain the threshold for the physical and biological quality indices from a cell suspension V/V^*, P/P^* and their yield. After analyzing the anomaly score of the stable phase, 0.72, 0.61

and 0.60 were set as the threshold for detecting outliers in V/V^*, D/D^* and yield measurements after filling process with mixing, respectively.

3.2.3. Stability and instability indices

The stability and instability of a process is quantified by measuring variability in the output. There are many conventional statistical methods for measuring variability, these include variance, standard deviation, range, average absolute deviation, median absolute deviation, interquartile range etc. [61,85]. Based on the data's underlying characteristics, any of the above-given statistics can be used for measuring the variability. The coefficient of variation (CV) derived from the ratio of standard deviation over the average has been used as an indicator for variability in many industries [55,86,87]. This statistic has served as a reliable tool to quantify the stability of a process and in turn, used for process control. A process can be considered unstable if the CV goes beyond the set limits. A prerequisite to use CV is for the data to be normally distributed; however, this is not observed in biological data [61]. Therefore, the classical CV might not be a robust estimator of variability when measuring the process stability of stem cells-based filling process. This drawback can be circumvented if the outliers in the data are detected and avoided in the stability analysis. The proposed CV for stability (stability index) avoids the outliers while measuring the CV, after outliers are detected with Isolation Forest algorithm. Once the stability index is quantified, it can be subtracted from the overall CV to get the CV from instability.

Overall CV = $\frac{s\ (standard\ deviation)}{\overline{x}\ (mean/average)}$

CV for stability = $\frac{s\ (standard\ deviation\ without\ outliers)}{\overline{x}\ (mean\ without\ outliers)}$

CV for instability = Overall CV – CV for stability

3.3.Materials and methods

3.3.1. Data acquisition for (V/V^*, D/D^*, P/P^*)

Data acquisition for each quality index was done exactly as described in chapter two.

3.3.2. Calibration of peristaltic pump

Data acquisition for each quality index was done exactly as described in chapter two.

3.4. Results

3.4.1. Algorithm for analyzing fluctuations in a batch and variation between batches

The algorithm analyzes fluctuations in a batch and classifies them as filling-order-dependent and random fluctuations. It uses Isolation Forest outlier detection to detect outlier fluctuations in a batch based on the threshold for outlier classification described the previous section. If more outliers are detected at a sample order, then the fluctuation at that location is declared filling-order-dependent. Five batches were analyzed in this study. If outliers were detected more than three times for each sample location, order-dependent fluctuation at that location was confirmed. Otherwise, it was classified as a random fluctuation. After the outlier classification, the CV for stability δ and CV for instability δ` are quantified to understand fluctuations in a batch. Variations between batches were also quantified with $\delta$ and $\delta$`. The algorithm is depicted in **Figure** 3.7.

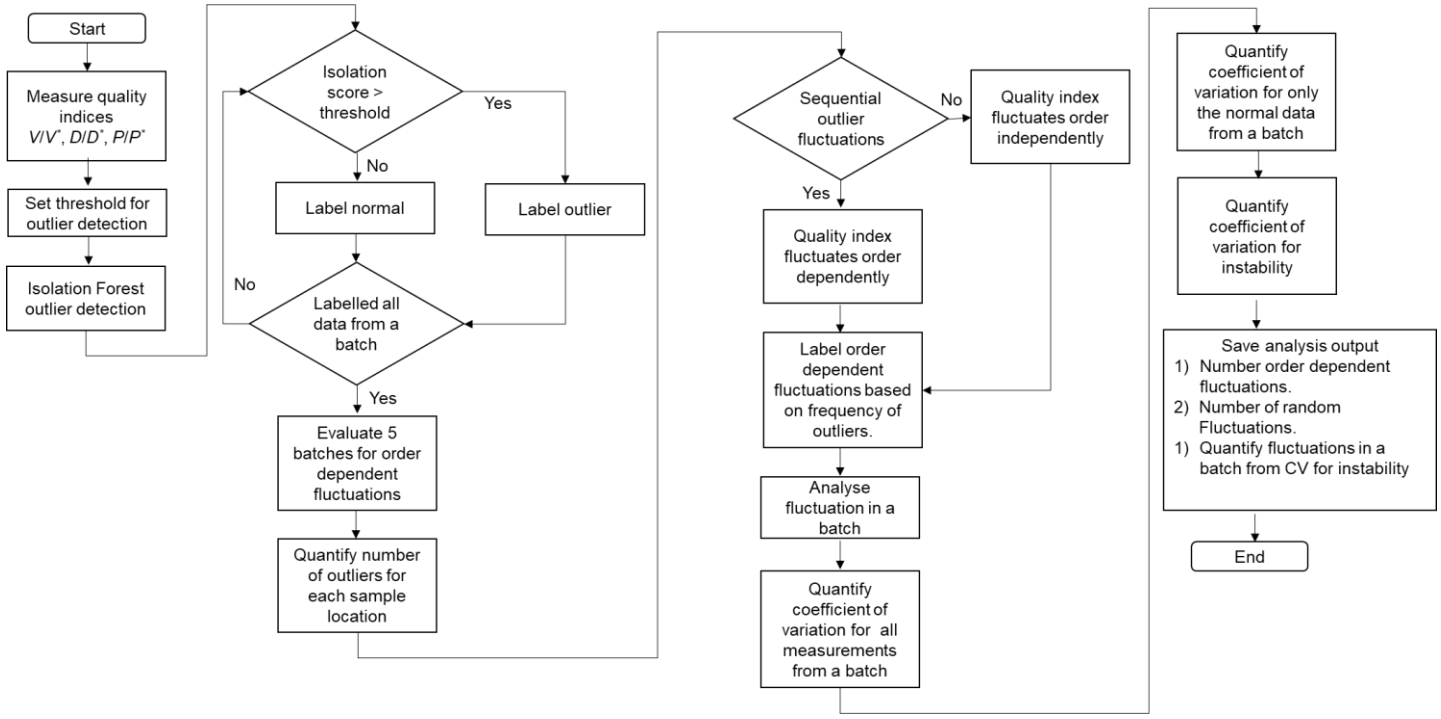


Figure 3.7 Algorithm for the analysis of fluctuations within a batch. The algorithm categorizes fluctuations within a batch as order-dependent and random fluctuations. After classification CV for stability and CV for instability is quantified.

3.4.2. Order-dependent fluctuation of the quality indices

The fluctuations in the batches for the quality indices and viability indices are presented in **Figures** 3.8. to 3.12 V/V^* was determined to have order-dependent (as more than once an outlier was detected at the same sample location in different batches) fluctuations in the final sample for the filling processes with and without the mixing operation (this was observed regardless of the nature of input material, fill volume always had similar trend). Vial No. 100 in all five batches (while using solution as the input material) after filling with the mixing operation was classified as an outlier, whereas only three out of five batches had an outlier Vial No. 100 for the filling process without the mixing operation as depicted in **Figure** 3.8. **Figure** 3.9 shows V/V^* when the input material is a suspension. D/D^* was determined to have order-dependent fluctuations for the final two samples (Vial Nos. 95 and 100) after the filling process without mixing. Samples (Vial No. 100) from four batches and (Vial No. 95) from three out of five batches were classified as outliers as shown in **Figure** 3.10A. Outlier frequency of Yield (Y) for the filling process with suspension as input material is shown in **Figure** 3.10B. Order-dependent fluctuations and random fluctuations can be observed. It can be noted that the order dependence in Y for filling with mixing and without mixing are coming the interaction of V/V^* and D/D^* with the mixing operation. No order-dependent fluctuations were observed for quality index P/P^* and Y associated with cell suspension, as shown in **Figure** 3.11. Random fluctuations were observed for all three quality indices. **Figure** 3.12 shows the trends of viability indices γ, β, α, that are used to calculate cell potential. Only random fluctuations were observed for the viability indices.

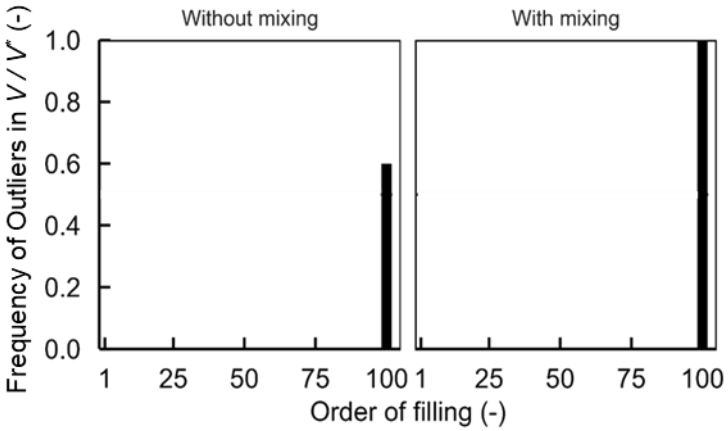


Figure 3.8 Outlier frequency for physical quality indices V/V^* from a system with solution as input material. Five batches for each quality index were evaluated.

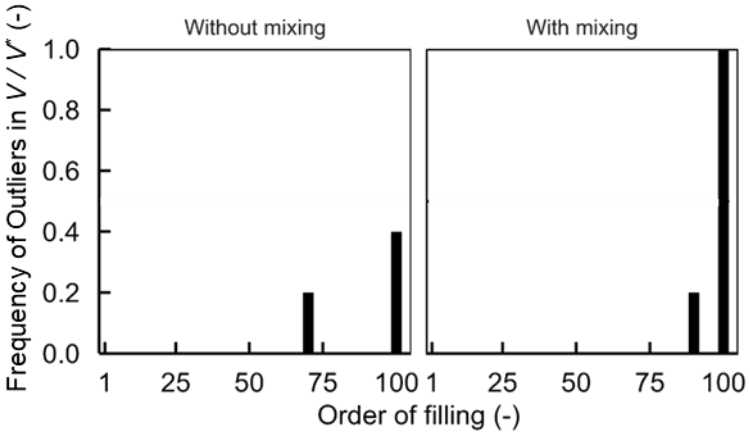


Figure 3.9 Outlier frequency for physical quality indices V/V^* from a system with suspension (polystyrene beads in cryoprotectant medium) as input material. Five batches for each quality index were evaluated.

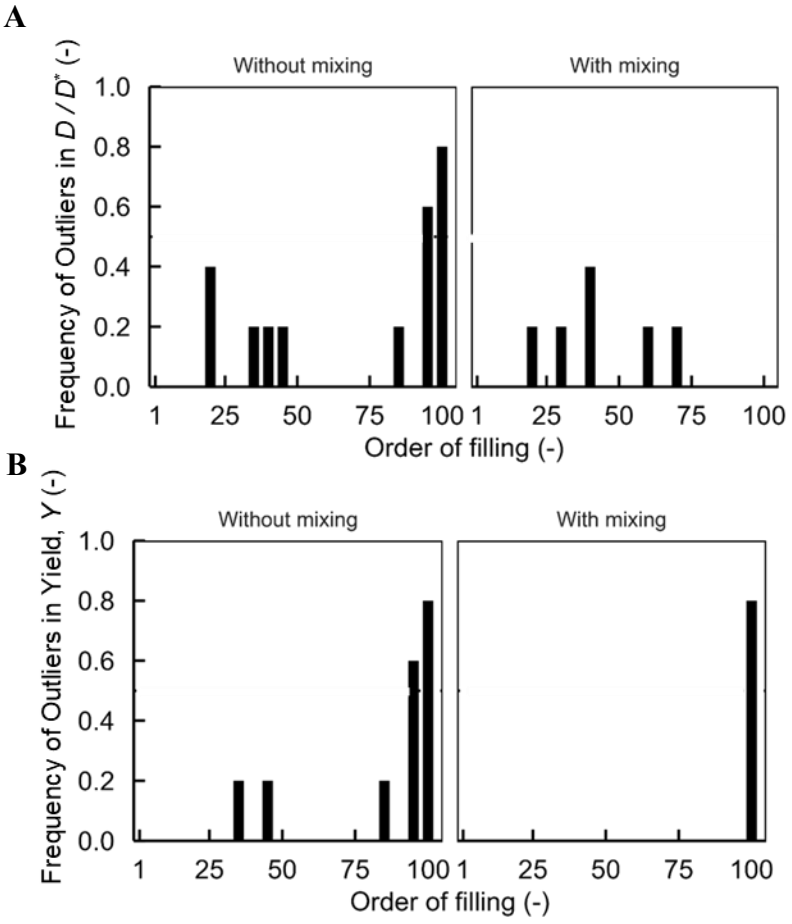


Figure 3.10 A) Outlier frequency for physical quality index D/D^* from a system with suspension (polystyrene beads in cryoprotectant medium) as input material. B) Outlier frequency in Yield (Y) from a system with suspension as input material. Y was calculated as a product of V/V^* and D/D^*. Five batches were evaluated.

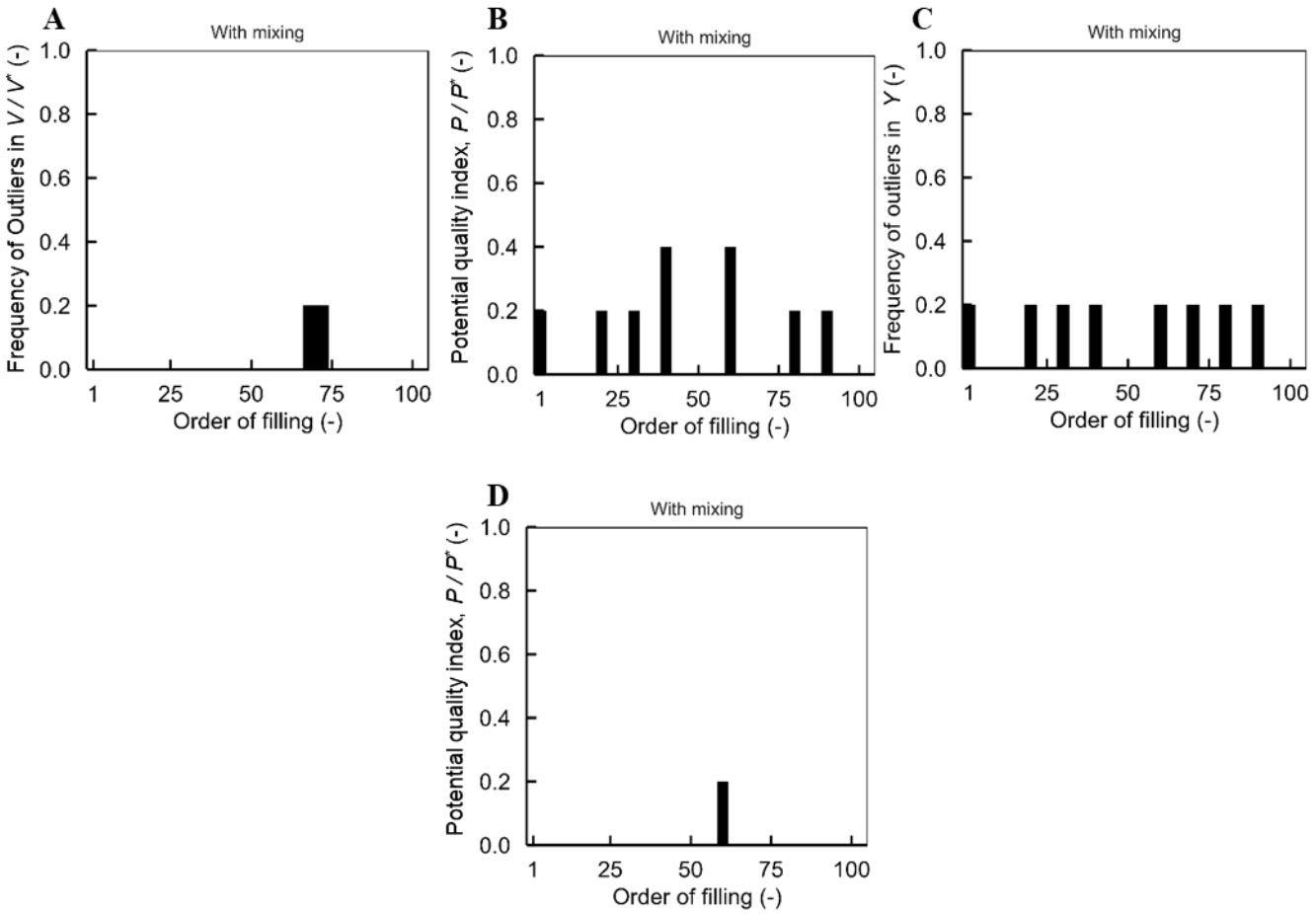


Figure 3.11 A) Outlier frequency for V/V^* from a system with cell suspension (hiPSCs in cryoprotectant medium) as input material. B) Outlier frequency for P/P^* C) Outlier frequency for Yield (Y). Random fluctuations were observed in both V/V^* and P/P^*. D) Frequency of outliers in P/P^* for a filling process at 4° C. Five batches were evaluated.

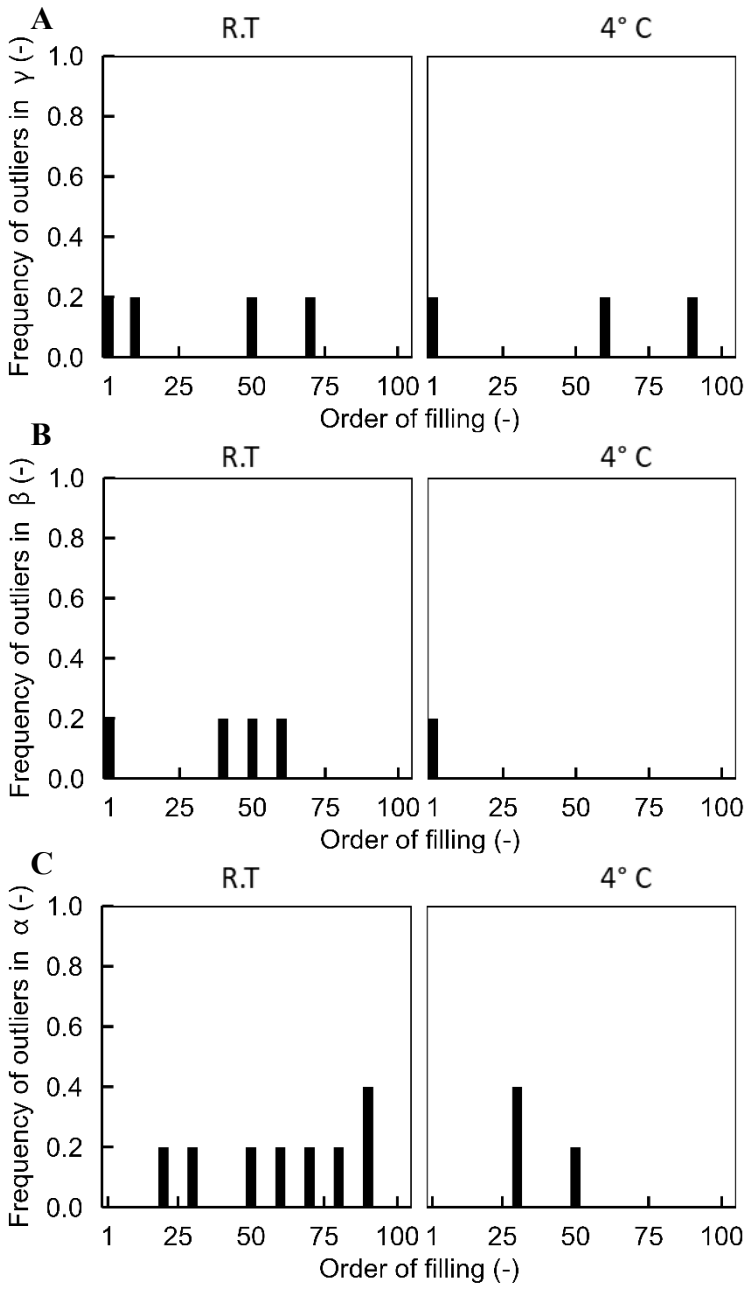


Figure 3.12 Outlier frequency for the viability indices. A) survival ratio (γ) for filling process at room temperature and 4° C. B) recovery ratio (β) for filling process at room temperature and 4° C. C) attachment efficiency (α) for filling process at room temperature and 4° C. Five batches were evaluated.

3.4.3. Fluctuation within batch analysis

After the quality indices data was categorized as normal and outlier based on the threshold limit, CV for stability (δ) and CV for instability (δ`) can be calculated. These indices are used to quanitify the fluctuations within a batch. The quality indices and Yield (Y) are measured depending on the input material, after which the stability and instability are estimated. **Figure** 3.13 shows the fluctuations in V/V^* after the filling process without and with mixing. The average δ for this process was close to 0.01 and the average δ`was estimated to be 0.03 and 0.16 for the filling process without and with mixing, respectively. The values for each of the five batches are summarized in **Table** 3.1.

Figure 3.14 shows the fluctuations in V/V^*, D/D^* and Yield (Y) after the filling process of a suspension without mixing operation. The average δ for this process was close to 0.10 and the average δ`was estimated to be 0.03. The values for each of the five batches are summarized in **Table** 3.2. **Figure** 3.15 shows the fluctuations in V/V^*, D/D^* and Yield (Y) after the filling process of a suspension with mixing operation. The average δ for this process was close to 0.04 and the average δ`was estimated to be 0.08. The values for each of the five batches are summarized in **Table** 3.3.

Figure 3.16 shows the fluctuations in V/V^*, P/P^* and Yield (Y) after the filling process of cell suspension (hiPSCs in cryoprotectant) with mixing operation. Only 10 samples were analyzed due to the difficulty in handling multiple samples and performing cell viability assays while minimizing the influence of sample handling time on the cells. The final vials of a batch were not measured as they did not contain enough cell suspension for performing

cell viability assays. The average δ for this process was close to 0.07 and the average δ`was estimated to be 0.04. The values for each of the five batches are summarized in **Table** 3.4. **Figure** 3.17 compares the normal cell suspension filling process performed at room temperature against the filling process performed at 4° C. The average δ for the filling process at room temperature and 4° C were 0.07 and 0.06, respectively. The δ` were 0.04 and 0.01, respectively. The values for each of the five batches are summarized in **Table** 3.5. Similarly, viability indices and their fluctuation within batches for the filling process at room temperature and 4° C are given in **Figures** 3.18 to 3.20.

Table 3.1. Analysis of fluctuations within a batch for filling process with solution as input material. *V/V**

		Overall CV	CV for stability	CV for instability	Number of outliers
Without mixing	Batch 1	0.18	0.01	0.18	1
	Batch 2	0.01	0.01	0.00	1
	Batch 3	0.01	0.01	0.00	0
	Batch 4	0.01	0.01	0.00	1
	Batch 5	0.01	0.01	0.00	0

		Overall CV	CV for stability	CV for instability	Number of outliers
With mixing	Batch 1	0.18	0.01	0.17	1
	Batch 2	0.08	0.01	0.07	1
	Batch 3	0.16	0.01	0.15	1
	Batch 4	0.21	0.01	0.20	1
	Batch 5	0.22	0.01	0.21	1

Table 3.2. Analysis of fluctuations within a batch for filling process using suspension as input material. *D/D**

		Overall CV	CV for stability	CV for instability	Number of outliers
Without mixing	Batch 1	0.11	0.08	0.03	2
	Batch 2	0.18	0.15	0.03	1
	Batch 3	0.14	0.10	0.04	3
	Batch 4	0.10	0.05	0.05	5
	Batch 5	0.10	0.08	0.02	2

Table 3.2. Analysis of fluctuations within a batch for filling process using suspension as input material. Yield (*Y*)

		Overall CV	CV for stability	CV for instability	Number of outliers
Without mixing	Batch 1	0.11	0.08	0.03	2
	Batch 2	0.18	0.15	0.03	1
	Batch 3	0.14	0.10	0.04	3
	Batch 4	0.10	0.05	0.05	5
	Batch 5	0.10	0.08	0.02	2

Table 3.3. Analysis of fluctuations within a batch for filling process using suspension as input material. D/D^* and Yield (*Y*)

		Overall CV	CV for stability	CV for instability	Number of outliers
With mixing	Batch 1	0.11	0.08	0.03	2
	Batch 2	0.18	0.15	0.03	1
	Batch 3	0.14	0.10	0.04	3
	Batch 4	0.10	0.05	0.05	5
	Batch 5	0.10	0.08	0.02	2

		Overall CV	CV for stability	CV for instability	Number of outliers
With mixing	Batch 1	0.11	0.08	0.04	2
	Batch 2	0.18	0.16	0.02	1
	Batch 3	0.14	0.11	0.02	2
	Batch 4	0.11	0.07	0.04	3
	Batch 5	0.10	0.08	0.02	2

Table 3.4. Analysis of fluctuations within a batch for filling process using suspension as input material. P/P^* and Yield (Y)

		Overall CV	CV for stability	CV for instability	Number of outliers
With mixing	Batch 1	0.10	0.07	0.03	2
	Batch 2	0.09	0.08	0.01	2
	Batch 3	0.09	0.06	0.03	2
	Batch 4	0.10	0.05	0.05	2
	Batch 5	0.11	0.05	0.06	2

		Overall CV	CV for stability	CV for instability	Number of outliers
With mixing	Batch 1	0.11	0.06	0.04	2
	Batch 2	0.09	0.09	0.01	1
	Batch 3	0.10	0.08	0.02	1
	Batch 4	0.10	0.05	0.06	2
	Batch 5	0.11	0.05	0.06	2

Table 3.5. Analysis of fluctuations within a batch for filling process using suspension as input material. P/P^* at 4° C.

		Overall CV	CV for stability	CV for instability	Number of outliers
With mixing	Batch 1	0.04	0.03	0.01	1
	Batch 2	0.09	0.09	0.0	0
	Batch 3	0.04	0.04	0.00	0
	Batch 4	0.06	0.06	0.00	0
	Batch 5	0.08	0.08	0.00	0

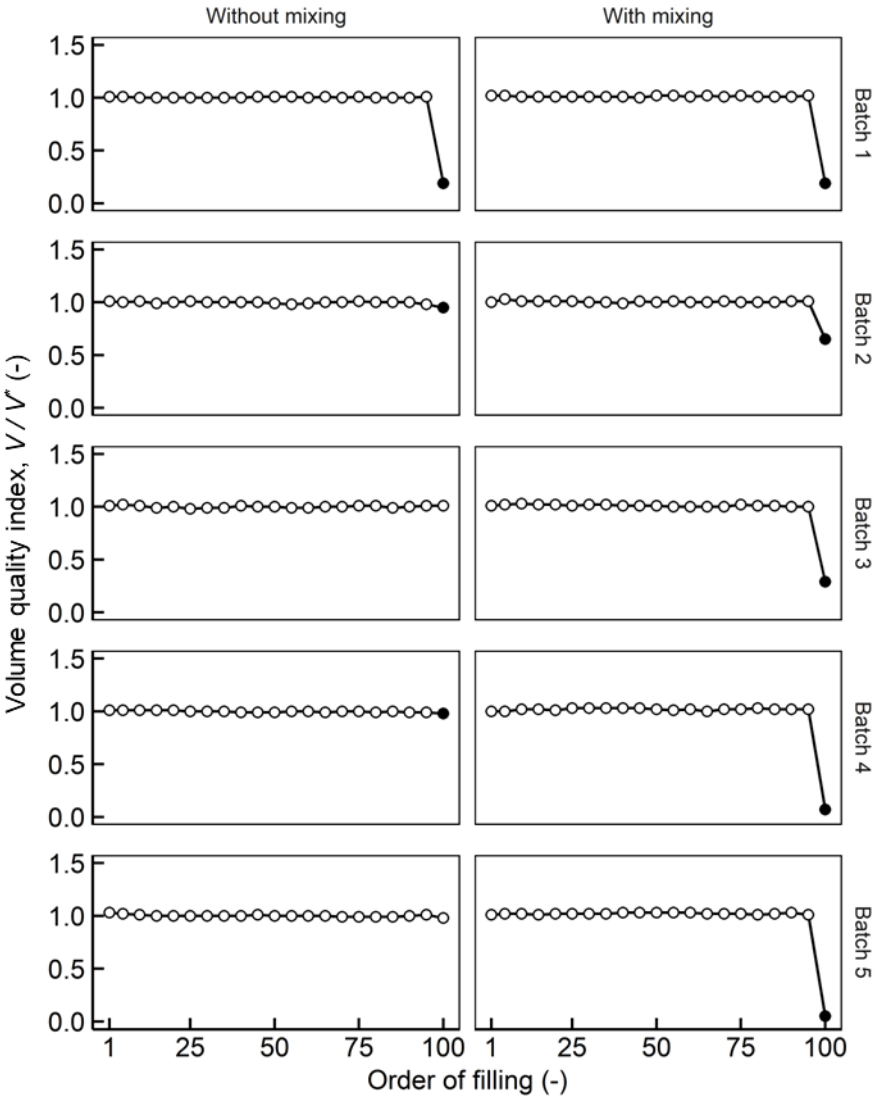


Figure 3.13. Analysis of fluctuations within a batch for filling process with solution as input material. V/V^* is measured and analyzed to estimate CV for stability (δ) and CV for instability (δ`). Here, V/V^* after filling process without and with mixing operation is measured and analyzed. Five batches were evaluated. Open circles represent normal data points and closed circles represent outlier data.

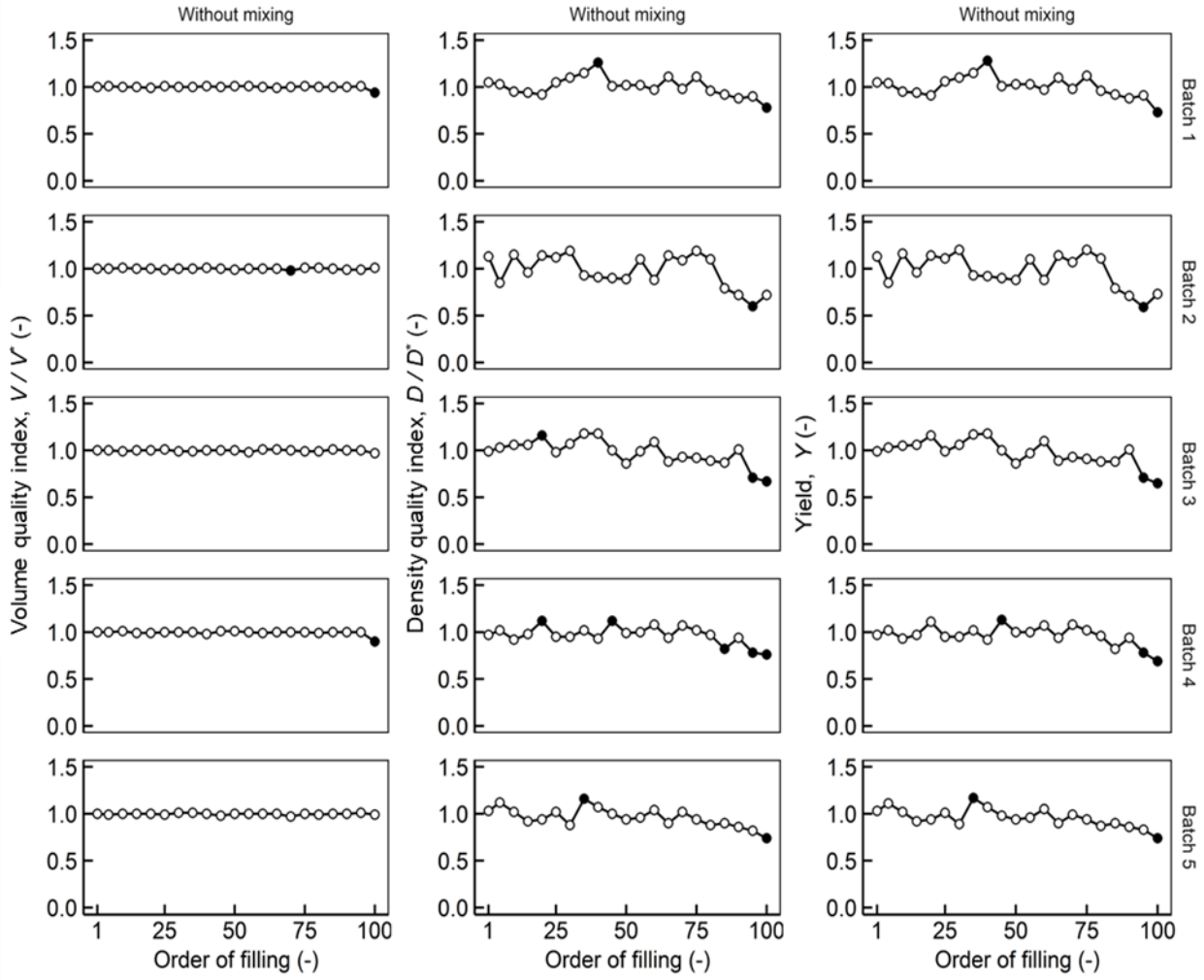


Figure 3.14 Analysis of fluctuations within a batch for filling process with suspension (polystyrene beads in cryoprotectant media) as input material. V/V^*, D/D^*, and Y are measured and analyzed to estimate CV for stability (δ) and CV for instability (δ`). Here, the filling process without mixing operation was performed. Five batches were evaluated. Open circles represent normal data points and closed circles represent outlier data.

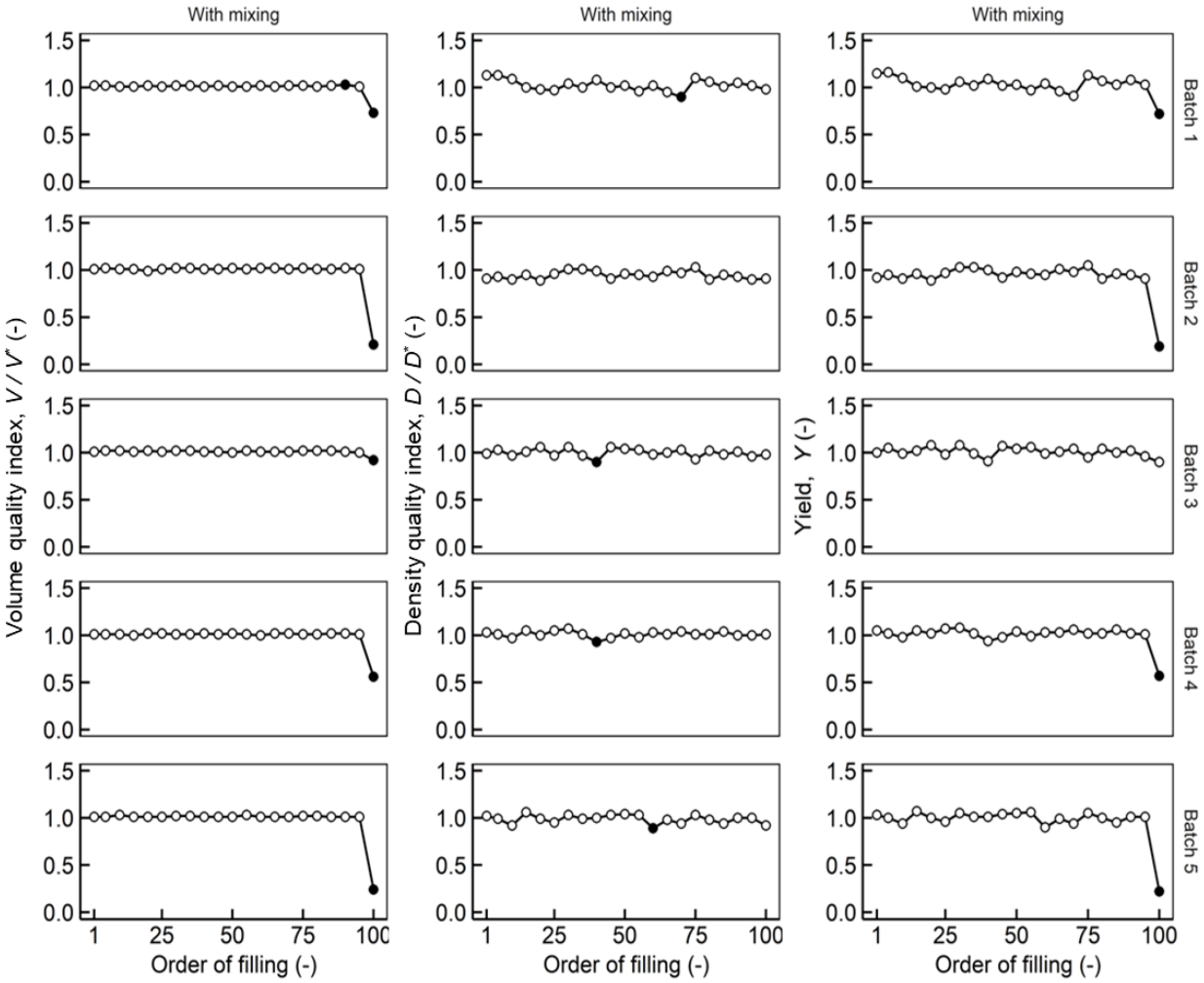


Figure 3.15 Analysis of fluctuations within a batch for filling process with suspension (polystyrene beads in cryoprotectant media) as input material. V/V^*, D/D^*, and Y are measured and analyzed to estimate CV for stability (δ) and CV for instability (δ`). Here, the filling process with mixing operation was performed. Five batches were evaluated. Open circles represent normal data points and closed circles represent outlier data.

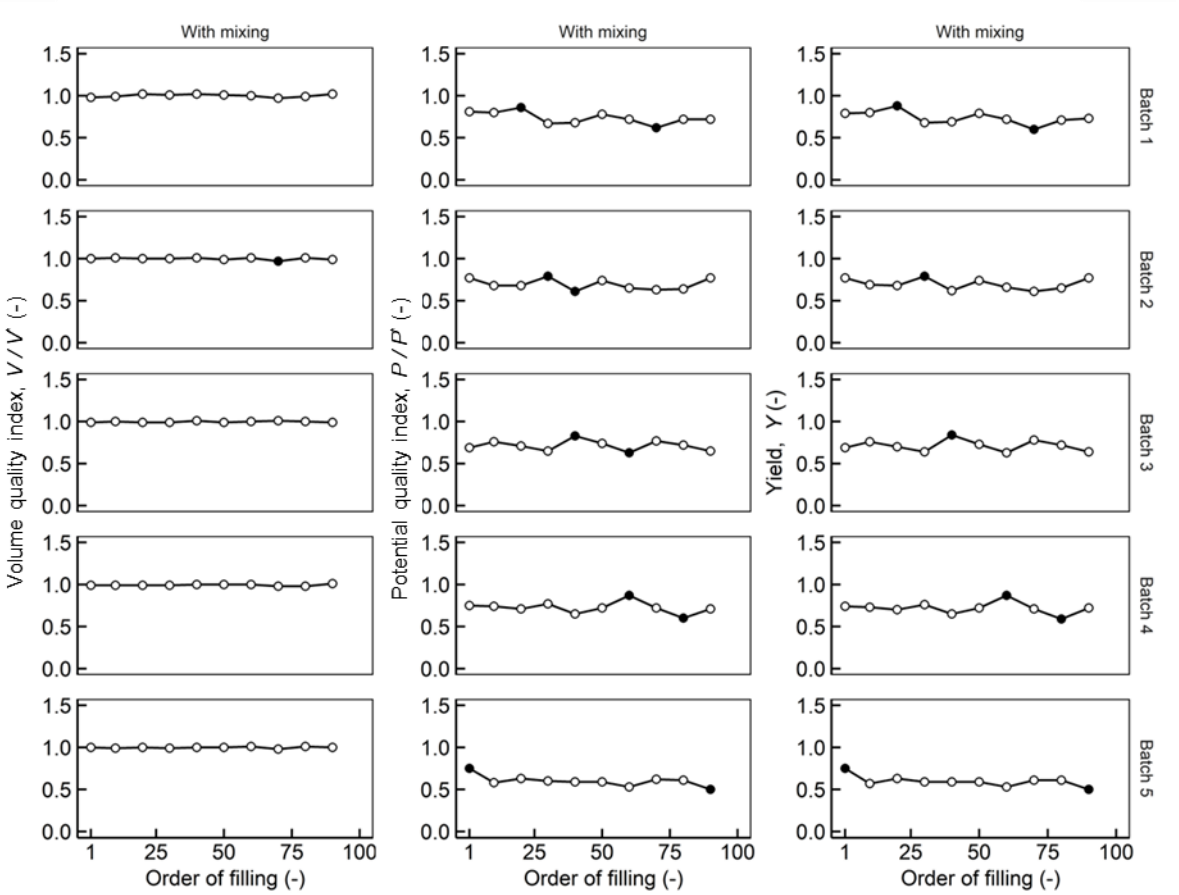


Figure 3.16 Analysis of fluctuations within a batch for filling process with cell suspension (hiPSCs in cryoprotectant media) as input material. V/V^*, P/P^*, and Y are measured and analyzed to estimate CV for stability (δ) and CV for instability (δ`). Here, the filling process with mixing operation was performed. Five batches were evaluated. Open circles represent normal data points and closed circles represent outlier data.

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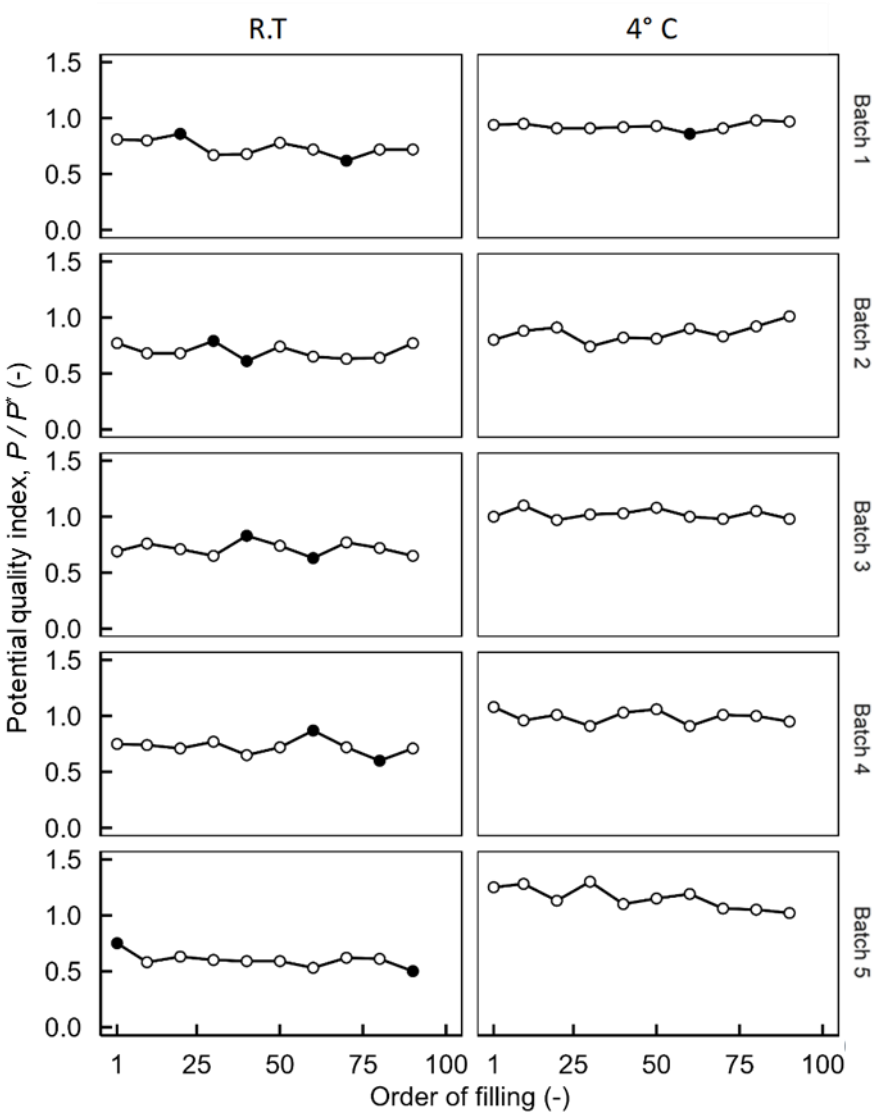

Figure 3.17 Comparison of fluctuations within a batch for P/P^* after filling at room temperature and 4° C. Open circles represent normal data points and closed circles represent outlier data.

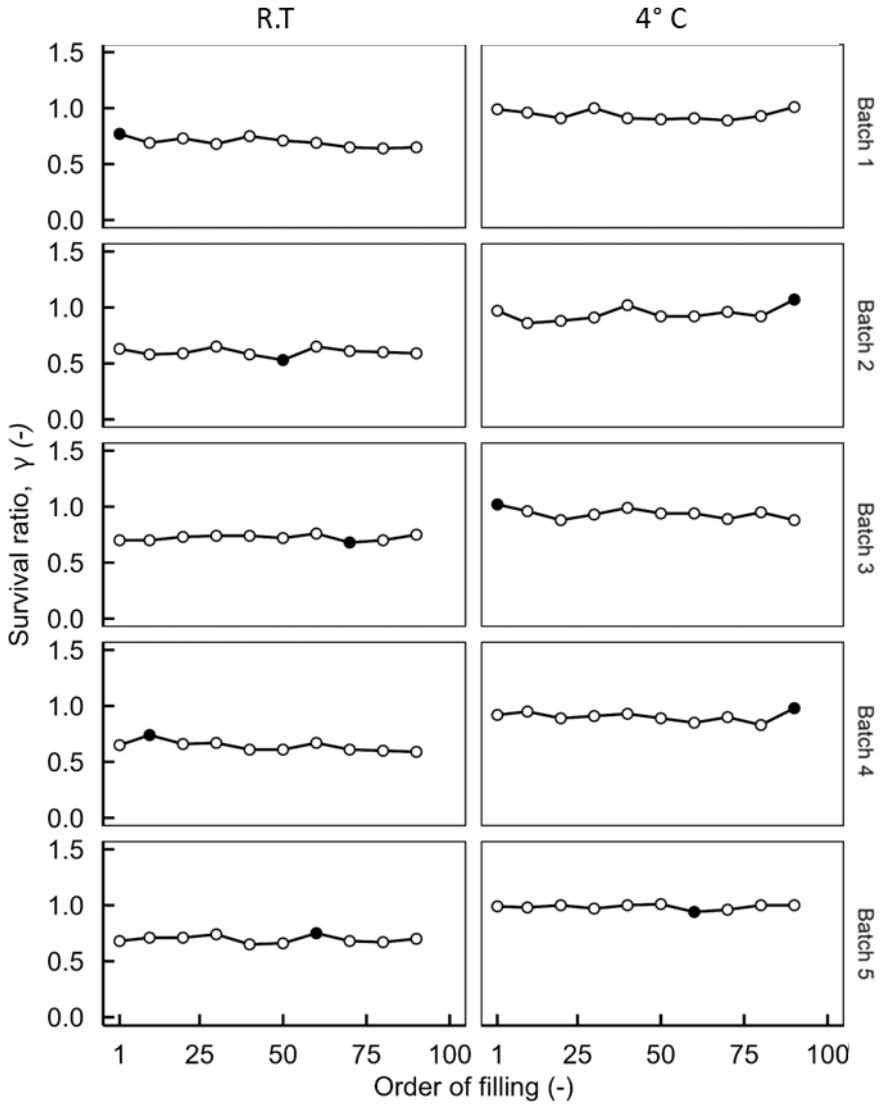

Figure 3.18 Comparison of fluctuations within a batch for survival ratio (γ) after filling at room temperature and 4° C.

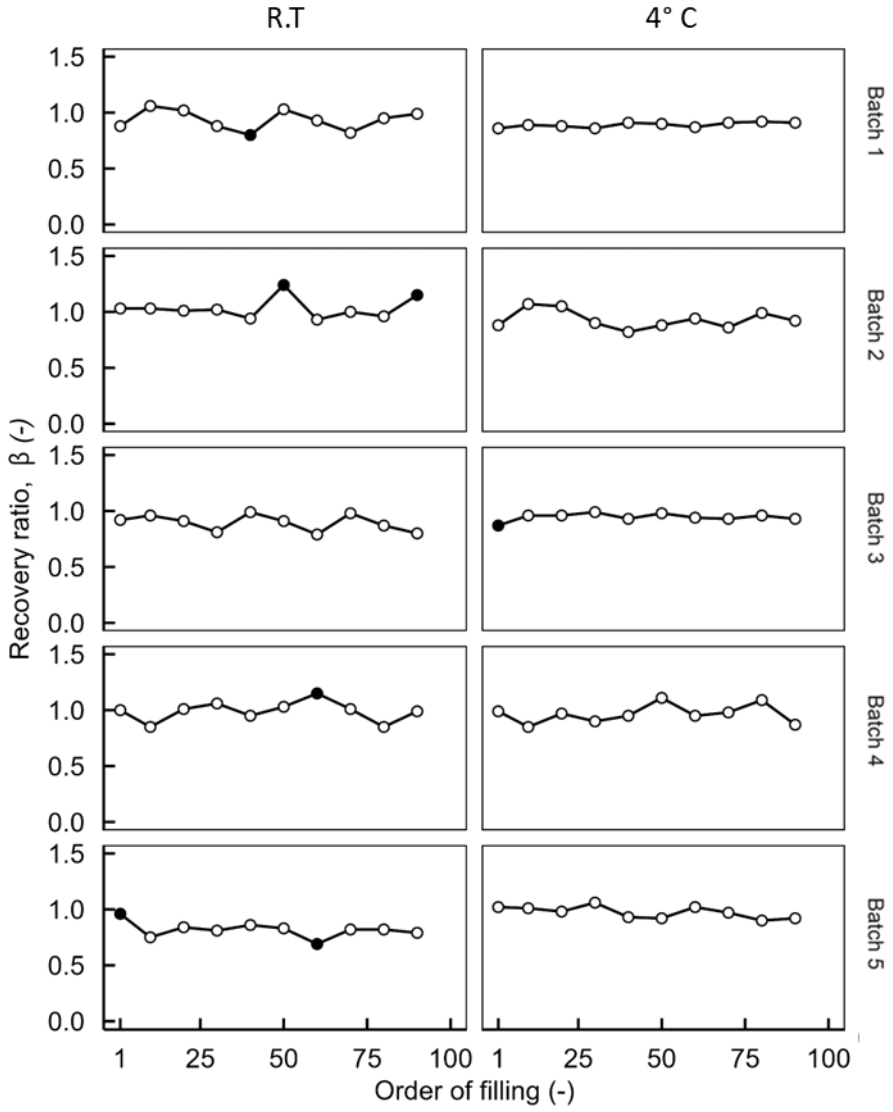

Figure 3.19 Comparison of fluctuations within a batch for recovery ratio (β) after filling at room temperature and 4° C.

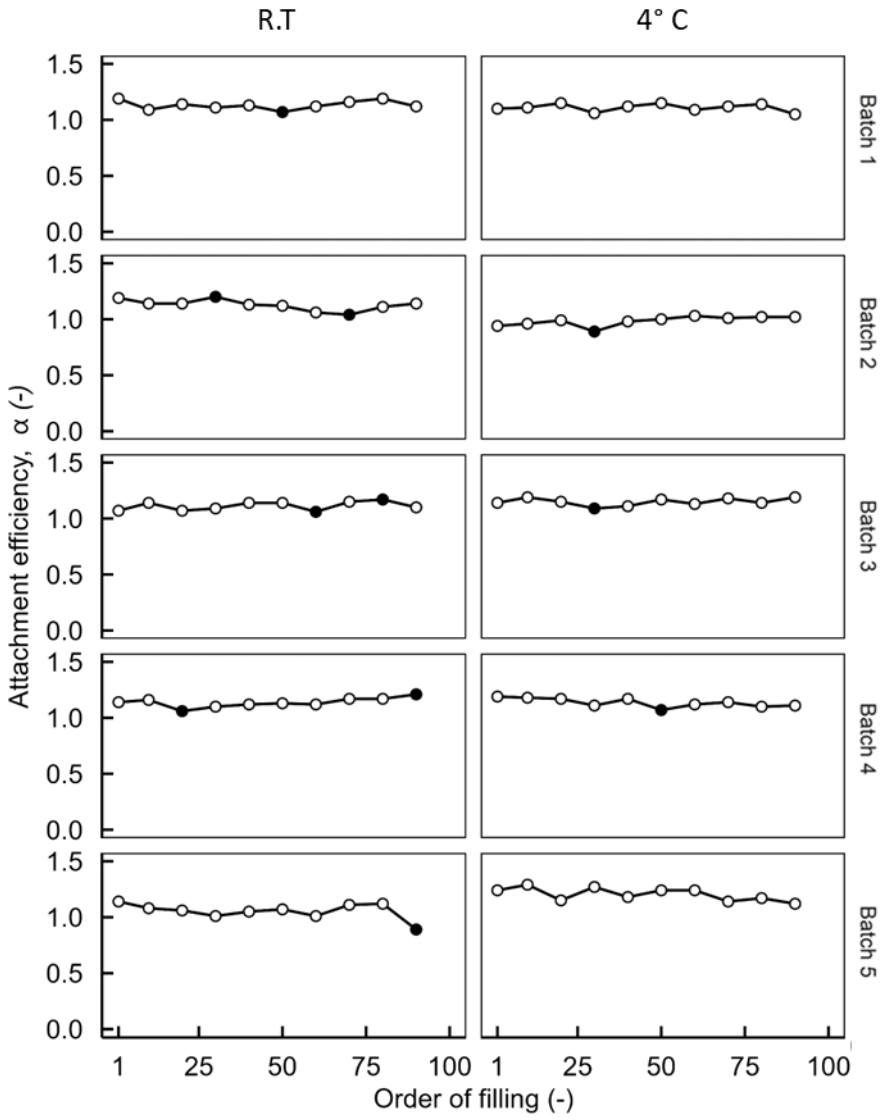

Figure 3.20 Comparison of fluctuations within a batch for attachment efficeincy (α) after filling at room temperature and 4° C

3.4.4. Variation between batches

Variations between batches for the quality indices were analyzed after classifying batches as either normal or outliers. Unlike the analysis of fluctuations in a batch, where the classification was done based on the establishment of threshold values derived from a stable phase within a batch, the methodology for classifying batches as normal or outlier follows the conventional fixed threshold value for all the quality indices. In this case, 0.6 was selected as the threshold for the anomaly score, and batches with an average value having an anomaly score of more than 0.6 were classified as outliers. Ten batches were analyzed for all the quality indices and compared to estimate the variation of each between batches. The physical quality indices had lower variation between batches as expected (for the filling process with mixing). This outcome is intuitive as they also have a lower degree of fluctuations within a batch. The variation between batches for P/P^* is relatively high compared to the physical quality indices. The strategy to mitigate this using a 4° C filling process does not seem to do much improvement. The variations still exist between batches, but it does improve the overall average cell potential. **Figure** 3.21A gives a detailed view of the variation between batches for V/V^*. **Figure** 3.21B gives a detailed view of the variation between batches for D/D^*. Figures 3.21C and 3.21D give a detailed view of variation between batches for P/P^* at room temperature and 4° C. The variation between batches is summarized in **Figure** 3.21 E.

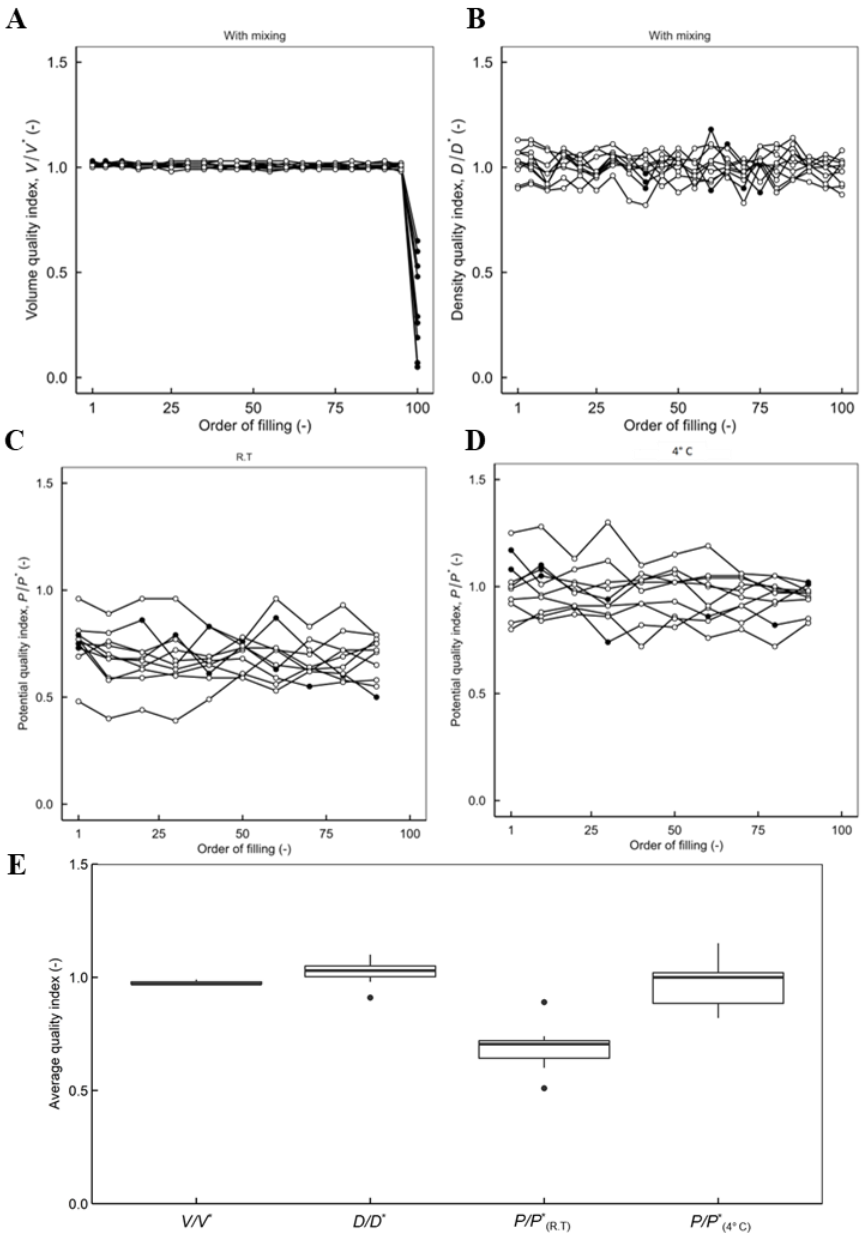

Figure 3.21. Variation between batches for each quality index was measured to compare the stability of the filling process. A) variation of V/V^* between batches for filling process with mixing operation. B) variation of D/D_* between batches for filling process with mixing operation. C) variation of P/P^* between batches for filling process with mixing operation at room temperature D) variation of P/P^* between batches for filling process with mixing operation at 4° C. E) comparison of average quality index between batches, filled circles indicate batches with outlier average values for each quality index. Ten batches were evaluated.

3.5. Discussion

Conventional SPC utilizes parametric analysis for detecting these outlier measurements, but as previously stated, this requires a better understanding of the underlying interaction between the process unit operations and the process output. This type of analysis is called limit sensing or limit value checking [62]. Statistical tools like control charts are examples of this approach; when the measured quality indices are between the limits of the model, the process is said to be under statistical process control [71,88]. If any measurement is detected to be outside the limits, a diagnosis for the process is performed, and feedback is generated for process control and improvement. The Gaussian distribution (normal distribution) is used as the underlying statistical model to monitor and control the variation in the output. These statistical tools cannot accurately analyze measurements that do not follow a normal distribution. Moreover, multivariate analysis is also challenging with these conventional techniques. Nonparametric statistical tools are an alternative to these conventional analysis techniques, and with the advent of advanced machine learning algorithms, the limitations of conventional techniques are being resolved [66,72] .

The heterogeneity of the output can be quantified to understand the process stability or instability. The current study categorizes the process instability into fluctuations within a batch and variations between batches. Analysis of process instability is done by detecting outlier measurements of quality indices. Isolation Forest, a machine learning algorithm, is used to detect outlier measurements [63]. The technique is nonparametric and, therefore, robust against the effects of outliers on the analysis. The algorithm has been deployed in

various fields and proven its capabilities with both simple and complex data sets, including high-dimensional data (a common feature of biological measurements) [82]. Generally, the data with the anomaly scores close to unity is classified as outliers in an unsupervised analysis. Therefore, the threshold for outlier classification can vary on a case-to-case basis depending on the inherent nature of the entity that the data represents. Physical attributes that are not subject to unpredictable changes are easier to measure and analyze compared to biological attributes, where the measurements are highly time-dependent and sensitive to environmental cues. This characteristic of biological data makes it difficult to set a threshold for categorizing normal from outlier data. In the current study, we proposed a method for establishing the threshold of outlier detection based on Isolation Forest anomaly scores. After a stable phase was defined for each of the quality indices that were proposed in Chapter Two, the filling process was performed, and the indices were measured.

The highest anomaly score from the stable phase measurements is selected as the threshold for detecting outliers. The anomaly scores for the stable phase measurements were used as a reference standard for an entire batch, ensuring consistency between the stable phase and rest of the batch. Ten vials (from 5 to 14) were declared to be the stable phase vials for all the quality indices and the threshold for anomaly score were selected. This logic of classifying data based on a specific threshold value defined by the user is similar to supervised classification. Therefore, in the current study, we converted the unsupervised outlier detection algorithm to a supervised algorithm based on a user-defined stable phase. This unique approach gives custom threshold values for each quality index based on their respective stable phase measurements.

Moreover, even if the quality index is an amalgamation of various other indices (potential quality index is a product of viability indices, yield is a product of two or more quality indices), the algorithm can handle multidimensional data. The outlier threshold was observed to be closer to unity for the stable phase of physical quality indices; this was true for V/V_* after the filling process with and without mixing. The D/D^* index followed a similar trend after the process was stabilized with the mixing operation. This threshold value was lower than physical quality indices for cell potential P/P^* where the inherent variation between samples is high. There is a clear indication that if a system is more stable, then the threshold for outlier detection would be closer to unity and vice versa. It also becomes intuitive that detecting an outlier among physical quality index measurements is easier than the biological quality index.

Once the data is categorized into normal and outlier, process stability and instability are quantified using the CV for stability (δ) and CV for instability (δ`). The coefficient of variation is a classic statistic used to measure the variation associated with measurements and has been extensively used in engineering sciences for reliability analysis. The problem with directly using this statistic in understanding complex biological data (or data with high unpredictability) is that it is highly susceptible to the influence of outlier data. CV for stability circumvents this by removing the data points that are outliers (based on the user-defined stable phase). Once δ is quantified, CV for instability can be calculated by subtracting δ from the overall CV.

After establishing the threshold for outlier detection, the algorithm and the proposed indices were used to quantify the fluctuations within a batch. Quality indices were measured and yield was calculated for the respective process output depending on the input material. Data from five batches were analyzed to understand filling order-dependent fluctuations as well as quantifying δ and δ`. The magnitude of $\delta$` gives an estimate of instability in the process at the same time, the magnitude of δ suggests the inherent process-related fluctuations.

As discussed in the results, V/V_* has a high fluctuation towards the end of a batch (vials 95-100). This can be attributed to fluctuations in fill volume due to air bubble formation in the input reservoir from mixing, peristaltic pump pulsation, changes in the input nozzle position, deterioration of the tubing and improper calibration of the pump. Any one of the above-stated phenomena or a combination of these can result in the fluctuations of V/V^*. In either case there seems to be order-dependent fluctuations in V/V^* towards the end of the batch. The mixing operation is observed to increase the fluctuation (process instability) in fill volume as observed in the greater number of outliers detected as well as the greater magnitude of δ`.

An intuitive approach would be to reduce the intensity of mixing or avoid the mixing operation altogether to decrease the instability due to the mixing operation. However, the mixing operation becomes crucial when the input material for the filling process is a suspension. It was observed in Chapter Two that particles in suspension sediment inside the reservoir as the process time increases. While filling a batch of suspension into vials, in the

absence of mixing operation, there is heterogeneity between the vials of the same batch. Since the sedimentation is random, the associated fluctuation in D/D^* of filled vials in a batch is relatively high. This fluctuation is more complex when the suspension is a cell suspension, where the cell potential degrades time-dependently. Therefore, avoiding the mixing operation for a filling process of a suspension is not recommended unless its homogeneity inside the input reservoir can be maintained.

For a suspension, the output has two quality indices that can be measured, the fill volume and the particle density. The yield is calculated as the product of V/V^* and D/D^*. The fluctuations in yield for the filling process without mixing is predominantly influenced by the fluctuations in D/D^*, while in case of the filling process with mixing, the fluctuations in yield is influenced by both V/V^* and D/D^*. Order-dependent fluctuations of D/D^* observed after the filling process without mixing are avoided in the filling process with mixing but this introduces order-dependent fluctuations in V/V^* measurements. Analysis after the filling process with and without mixing suggests how process instability is lowered, but there still seems to be instability in the process due to the mixing operation.

Cell suspension, compared to a suspension with physical beads, is more complex as the cells are dynamic systems that constantly interact with their environment [33–35]. The current study uses hiPSCs suspended in a cryoprotectant media. Cells experience osmotic shock and other mechanical stresses while being suspended, triggering various pathways that lead to cell death [54,80,89]. Cell potential quality index P/P^*, which is a product of viability indices, measures cells' viability and proliferative capacity after the filling process. The

filling process was always done with a mixing operation as it was necessary to maintain the suspension homogeneity before filling the cryovials to ensure cells were available for the viability assays after the filling process was finished. The fluctuations in yield are mainly consequences of fluctuations in cell potential. The final vails were not analyzed for cell suspensions as there was deficient fill volume for any further viability analysis. A decrease in the cell potential was seen after the filling process, this is consistent with the previous findings where it was suggested that hiPSCs suspended in cryoprotectant media become unviable with an increase in process time. Moreover, the fluctuations between samples are also greater and more unpredictable than the physical quality indices. After analyzing the viability indices, it can be further understood that survival ratio (γ) and attachment efficiency (α) are the most affected by the filling operation and tend to influence P/P^* the most. The mechanism of cell death corresponding to each viability assay phase has been elucidated previously. There are conditions that lead to immediate rupture of cells due to membrane integrity failure or it can have a tardive affect, where there is a delayed onset of cell death (DOCD) during the post-filling cell culture and expansion. The cell population in each sample of the filled vials will have specific routes to cell potential degradation due to intrinsic disorder within each cell and how individual cells respond to the environmental cues. Moreover, cell potential measurements are also affected by environmental noise.

Previous studies have suggested methods of improving cell viability during the filling process by targeting the various routes of cell death, mainly with the use of antioxidants and temperature control [48,54,89]. It was shown that reactive oxygen species (ROS) generated during the filling process leak from the mitochondrial chamber and start a cascade of

reactions that trigger apoptosis. Using antioxidants to remove the ROS or suppressing their overall generation by slowing the cell metabolism with low temperature has been shown to improve the overall cell viability and proliferative capacity. Based on this, the filling process for cell suspension was carried out at 4° C to lower the hiPSCs metabolic activity and maintain their cell potential near unity for an extended period. Although this strategy may improve the average cell potential, as seen from the previous studies, no evidence supports that they improve the overall process stability or decrease the instability. This lack of improvement in fluctuations within a batch was observed. The number of outlier measurements reduced with the 4° C filling process, but δ and δ` were not significantly different from the filling process at room temperature. Measuring the yield for cell suspension under temperature-controlled conditions was also difficult, as weighing the samples while maintaining the 4° C environment is challenging. It should also be noted that these measurements are highly time and stress-sensitive and, therefore, should be recorded with minimum delay as possible between samples [90,91]. The delay in recording/performing viability assays compounded with the intrinsic variation of cell population in each cell can influence the final cell potential measurements. The effect of 4° C on individual viability indices was also observed, consistent with previous studies.

The 4° C filling process seems to improve the average survival ratio, recovery ratio and attachment efficiency but fluctuations within the batch are not significantly reduced. This outcome suggests that other stress factors affecting the cells must be mitigated for the filling process to be more stable. Literature suggest that mechanical stress during filling process

(especially from the mixing operation) could be a major factor in process instability [92]. Future research should most likely target these issues to stabilize the process.

Analysis of fluctuations in a batch quantifies the differences between the samples of the same batch, while analysis of variation between batches looks into the differences between samples from a whole batch and another. Variation between batches needs to be controlled and minimized for reproducibility of the output. As with fluctuations within a batch, the process would be deemed unreliable if the variations between batches are not in control. Outlier measurements (that correspond to unstable fluctuations) were made after setting a threshold for outlier detection specific to each quality index and their corresponding yields. This approach, however, is not suitable for analyzing variation between batches. The present study, therefore, followed the conventional method of outlier classification based on interquartile ranges and boxplots to classify batches as normal or outliers [55,87,93]. After this, the quantification step follows the same principle as for fluctuations in a batch. δ and δ` were calculated for each quality index after analyzing 10 batches. As noted in the results, physical quality indices are much more stable between batches than biological quality indices. The variation between batches did not decrease for P/P^* even with a 4° C filling process (although the overall cell potential improved for the filling process at 4° C). This approach is similar to many other conventional stability analysis techniques that utilize a standard threshold value for outlier detection regardless of the nature of the underlying data.

3.6. Chapter summary

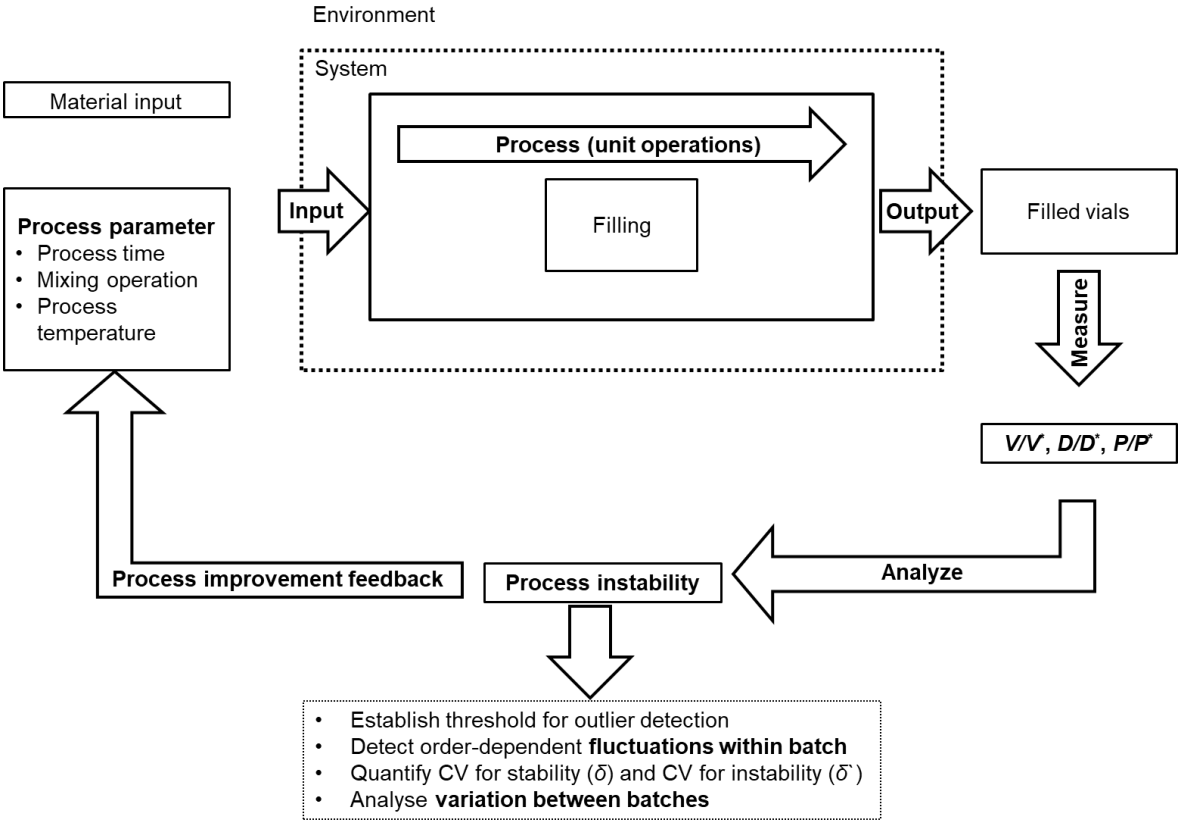

Figure 3.22. Process instability was characterized fluctuations within batch and variations between batches. Algorithm was designed specifically for the filling process using nonparametric outlier detection technique. The feedback from analysis was used to mitigate instability in the filling process.

This chapter proposed an analysis method that utilizes a nonparametric, unsupervised outlier detection algorithm (Isolation Forest) to quantify stability and instability in a filling process. The study proposed a new method for establishing the threshold of outlier classification for quality index measurements. The method for selecting the threshold for outlier classification transformed the general unsupervised outlier detection technique into a

supervised technique specific for filling. After establishing the threshold of outlier classification for each quality index, fluctuation within a batch and variation between batches for each of those quality indices are quantified and compared. Based on this assessment, the filling process stability for different types of input materials was compared. It was shown that the filling process was more stable for the physical quality indices. If not, it could be made more stable by introducing operations like mixing during the filling process. Cell potential after the filling process fluctuates more than physical quality indices even after implementing some improvement strategy. The present study's uniqueness comes from quantifying process stability and instability by establishing custom limits for outlier classification and analysis of fluctuations within a batch and variations between batches.

Chapter 4
General conclusion and future perspectives

This study focused on developing a method of analysis for the evaluation of process stability. A process needs to be stabilized before it can be improved, this makes stability analysis a crucial component of process design. A method of analysis suitable for handling different types of underlying data was the objective of this study. The first phase of research dealt with defining quality indices corresponding to certain attributes of the output of the filiing process. The output can have different quality indices corresponding to the nature of the input material used, for example, a homogenous solution can mainly be analyzed after the filling process by measuring just the fill volume. A suspension with a solid dispersed in a liquid dispersion phase will be analyzed after measuring the fill volume and the particle density. Similarly, a suspension (cell suspension) with particles that degrade dependent on process time/stress sensitive attributes will be analyzed after measuring the fill volume and the extent of degradation or viability of the components. In order to accomplish this goal, we proposed three quality indices, namely V/V^*, D/D^* and P/P^*. These indices were further used to calculate the yield from the filling process based on the nature of the input material by multiplying the quality indices. The second chapter focused on understanding these quality indices and how they behave after the filling process. We also focused on proper calibration techniques to achieve consistent filling techniques, which are crucial for the homogeneity of samples within and between batches. Once the trends of these proposed quality indices were observed, their relationship with process time was also analyzed. These interaction studies gave an in-depth understanding of the nature of the quality index. Moreover, the yield

calculated using these quality indices made the comparison of the filling process with different input materials possible.

Once the quality indices were proposed and their trends were observed after the filling process, the instability in the process must be quantified. The second half of this study focused on developing an instability quantification algorithm. Analysis of process stability during the process design phase is not a new concept and SPC has been used for this purpose. The conventional methods have shortcomings when handling datasets that are more complex and the nature of the underlying data is not known. In this study, a nonparametric unsupervised machine learning algorithm was selected to detect outlier measurements in process output as these are indications of process instability. The uniqueness of the study comes from the novel method of setting the threshold for outlier classification. Based on user input, a stable phase in the filling process is defined/designated and based on this stable phase, the outliers in the rest of the batch are identified. Outlier classification is the first step of instability analysis. After the classification, CV for stability (δ) and CV for instability (δ`) are calculated. In this way, the present study has transformed a conventional reliability analysis tool into a robust instability quantification method.

The process instability was further categorized into fluctuations within a batch and variations between batches. Filling order-dependnet fluctuations were confirmed in the case of V/V^* for both without and with mixing operation. Even though the mixing operation introduces some instability into the system, it cannot be avoided as the mixing operation is an important stabilizing mechanism during the filling process of suspensions. The mixing

essentially maintains the homogeneity of the material being dispensed into individual vials. Considering this information, the filling process was always done with mixing for cell suspensions.

The study further compares the fluctuations within a batch for different input materials under mixing and non-mixing conditions. As expected, physical quality indices had fewer fluctuations within a batch and even if high fluctuations were encountered, these were stabilized with mixing operation. Variation between batches was analyzed similarly, but the outlier detection algorithm used conventional fixed threshold value for classifying normal measurements from outlier measurements. Ten batches for each quality index were analyzed. As expected, the variation between batches for the physical quality indices was lower compared to the biological quality index. Based on the literature, improvement strategies were implemented to stabilize cell potential, utilizing a filling process at 4° C. This improved the average cell potential compared to the filling process at room temperature but did not mitigate the lack of stability. However, The analysis method was employed in different scenarios to evaluate the process stability. The results support its applicability in both normal filling and filling processes for cell suspensions. This proves that the developed method can be used for systems with different types of cells and different processes (not just limited to the filling process). The developed method can, therefore, be instrumental in process design for cell manufacturability.

Even though the method of instability analysis was robust and versatile for different types of input materials, the overall stability of the filling process with cell suspension was not improved even after implementing a 4° C filling process. This could be due to the sensitivity of cells to mechanical stress from the constant mixing operation [92,94]. This study never analyzed the impact of mixing on the cells' viability and proliferative capacity after the filling process. Multiple studies have implicated the negative impact of shear stress on cell viability and how it affects cell proliferation and differentiation potential of stem cells. The mixing operation during the filling process definitely exposes the cells in the suspension to shear stress for an extended period of time. This mixing operation cannot be avoided to maintain the homogeneity of the cell suspension. A future direction for this work could be elucidating the impact of mixing operation on the cell potential after the filling process and strategies to mitigate this effect. A low-shear system capable of maintaining the homogeneity of the input material for an extended period of time should be the ideal solution. Polymers like gellan gum has recently been supplemented in cell expansion media to alter the media rheology [94]. The media becomes a plastic fluid and particles suspended in it do not sediment, similar to normal suspensions. If such a system can be implemented in the filling process of hiPSCs, it is speculated that it may improve the process stability. Regardless, the method of analysis developed in this study could be used to assess the process stability of this new system by simply adjusting the threshold value for outlier detection based on the specific low-shear filling system.

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List of publications

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