



## Regular article

# Kinetic analysis of cell decay during the filling process: Application to lot size determination in manufacturing systems for human induced pluripotent and mesenchymal stem cells



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## ABSTRACT

Scale-up of a cell-manufacturing system involves an extension of the filling process time because of the increased number of vials to be filled with the cells suspended in a cryopreservation solution. As the solution includes cryoprotective agents such as dimethyl sulfoxide that is toxic to cells, a longer processing time has an adverse impact on the quality of cell products. Therefore, understanding the related cell-decay kinetics is a major challenge for the development of robust and scalable cell-manufacturing systems. In this study, a new set of performance indices were defined to quantify the state and potential of cells suspended in a cryopreservation solution, i.e., the survival ratio of cells suspended in cryopreservation solution was provided for the cell state estimation, and the recovery ratio of cells after freezing and thawing, the attachment efficiency of cells after seeding, and the specific growth rate of surviving cells were provided for the cell potential estimation. Using these indices, the time-dependencies of cell viability variation in human induced pluripotent (hiPSCs) and human mesenchymal stem cells (hMSCs) suspended in a cryopreservation solution were investigated. Based on the obtained kinetic data, strategies for developing and controlling cell-manufacturing systems were discussed.

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## 1. Introduction

Because of increased expectations for stem cell applications in regenerative medicine and drug discovery and development, rapid growth in the cell-manufacturing industry is predicted [1,2]. In response to increased demands for cell products and to achieve reductions in manufacturing costs, much effort has been focused on the scale up of cell expansion technologies [3]. As large-scale culture methodologies have matured upstream of the cell-production process, critical steps have shifted downstream [4–6]. For successful expansion in lot size, development of scalable downstream processes that enable high yield and homogenous production are required.

A typical downstream process consists of cell harvesting, clarification, concentration, formulation, filling and cryopreservation.

In larger scale production, the number of vials to be filled increases and the downstream process time is prolonged. When cells are preserved under cryogenic conditions, the cells are suspended in cryopreservation solutions, and in most cases, these include dimethyl sulfoxide (DMSO) as a cryoprotective agent to prevent intracellular ice formation during the freezing process [7]. While DMSO is known to be toxic to animal cells [8,9], there have been few reports regarding the quantitative evaluation of the relationship between cells' DMSO exposure time and viability reduction [10]. Therefore, comprehension of cell decay kinetics in cryopreservation solution is essential for enhancing cell manufacturability and developing robust and scalable cell-manufacturing systems. However, because of the intrinsic heterogeneity and uncertainty in cell populations, incomplete understanding of the biological characteristics of cell products, and technological difficulties in analyses, a new, specific, evaluation methodology was required to be developed for a cell-manufacturing system design [11].

In this study, a new set of performance indices relating to the cell state and potential in filling process were defined for the quanti-

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## Nomenclature

hiPSC	Human induced pluripotent stem cell
hMSC	Human mesenchymal stem cell
DMSO	Dimethyl sulfoxide
A	Cell population that survives after suspension, freezing and thawing, seeding and incubation
B	Intermediate injured cell population that survives suspension and freezing and thawing but dies during the seeding and incubation
C	Intermediate injured cell population that survives suspension but dies during freezing and thawing
D	Cell population that dies during suspension

## Indices

$\alpha$	Attachment efficiency of cells after seeding (–)
$\beta$	Recovery ratio of cells after freezing and thawing (–)
$\gamma$	Survival ratio of cells suspended in cryopreservation solution (–)
$\mu$	Specific growth rate of surviving cells ( $\text{h}^{-1}$ )
$k_d$	Rate constant of cell decay ( $\text{h}^{-1}$ )

## Parameters and variables

$A_c$	Acceptance criterion of relative viability (–)
[A]	Ratio of cells in status A against the total viable cells at $t_s = 0$ (–)
[B]	Ratio of cells in status B against the total viable cells at $t_s = 0$ (–)
[C]	Ratio of cells in status C against the total viable cells at $t_s = 0$ (–)
[D]	Ratio of cells in status D against the total viable cells at $t_s = 0$ (–)
$N_c$	Total cells per lot (cells)
$N_v$	Total number of vials per lot (vials)
$n_0$	Total viable cells at $t_s = 0$ (cells)
$n_{1,t_s}$	Total viable cells at $t_s$ (cells)
$n_{2,t_s}$	Total viable cells after $t_s$ of suspension and freezing and thawing (cells)
$n_{3,t_s}$	Total viable cells on the culture plate surface after $t_{c1}$ of incubation after $t_s$ of suspension and freezing and thawing (cells)
$n_{4,t_s}$	Total viable cells on the culture plate surface after $t_{c2}$ of incubation after $t_s$ of suspension and freezing and thawing (cells)
$V$	Volume of cell suspension per lot (L)
$t_a$	Available time for filling process (h)
$t_s$	Time cells are suspended in cryopreservation solution (h)
$t_{c1}$	Time cells are incubated after seeding for estimation of attachment efficiency (h)
$t_{c2}$	Time cells are incubated after seeding for estimation of specific growth rate (h)

tative evaluation of cell viability, and its time-dependent variation of human induced pluripotent (hiPSCs) and human mesenchymal stem cells (hMSCs) suspended in a cryopreservation solution were investigated. For the cell state estimation, the survival ratio of cells suspended in cryopreservation solution ( $\gamma$ ) was determined. In addition, for the cell potential estimation, the recovery ratio of cells after freezing and thawing ( $\beta$ ), the attachment efficiency of cells after seeding ( $\alpha$ ), and the specific growth rate of surviving cells ( $\mu$ ) were determined. The experimental kinetic data and the scalability of cell-manufacturing systems based on these data will be discussed.

## 2. Materials and methods

### 2.1. Culture of hiPSCs

The human induced pluripotent stem cell line Tic (provided by JCRB1331, JCRB Cell Bank, Osaka, Japan) was used in this study. The hiPSCs were maintained on iMatrix-511-coated ( $0.25 \mu\text{g}/\text{cm}^2$ ; Nippi Inc., Tokyo, Japan) cell culture dishes or flasks (Corning Inc., Corning, NY, USA) with commercially available medium (StemFit AK02N, Ajinomoto Co., Inc., Tokyo, Japan) at  $37^\circ\text{C}$  in air with 5%  $\text{CO}_2$ . For passaging, hiPSCs were detached by treatment with 5 mM EDTA/PBS(–) containing  $10 \mu\text{M}$  ROCK inhibitor Y-27632 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 10 min and then TrypLE Select (Thermo Fisher Scientific Inc., Waltham, MA, USA) with  $10 \mu\text{M}$  Y-27632 for 7 min at room temperature. After the above treatment, hiPSCs were detached by tapping or gentle flushing by pipette, transferred to centrifuge tubes, collected by centrifugation (180g, 3 min), and dissociated into single cells by pipetting in the previous medium. The dissociated cells were seeded at a density of  $2.5 \times 10^3$  or  $1 \times 10^4$  cells/ $\text{cm}^2$  into iMatrix-511-coated dishes or flasks with medium containing  $10 \mu\text{M}$  Y-27632. After one day of culture, the medium was changed to that without  $10 \mu\text{M}$  Y-27632, after which the medium was changed daily. After 4 or 5 days of culture, when the cells reached 80–90% confluency, the cells were passaged or used in the experiments.

### 2.2. Culture of hMSCs

The human mesenchymal stem cell line from bone marrow (abe2MCS, Lot No. 000037485; Lonza Ltd., Basel, Switzerland) was used in this study. The cells were maintained on cell culture dishes or in flasks (Corning Inc.) with Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Inc., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Life Technologies, Inc., Carlsbad, CA, USA) at  $37^\circ\text{C}$  in air containing 5%  $\text{CO}_2$ . For passaging, cells were detached by treatment with trypsin from porcine pancreas (Sigma Aldrich, Inc.) at  $37^\circ\text{C}$  for 5 min. After adding trypsin inhibitor (Wako Pure Chemical Industries, Ltd.), the cells were detached by tapping or gentle flushing by pipette and transferred to centrifuge tubes. After centrifugation (180g, 5 min), the cells were dissociated into single cells by pipetting in DMEM. The dissociated cells were then seeded at a density of  $5 \times 10^3$  cells/ $\text{cm}^2$  into dishes or flasks with DMEM supplemented with 10% FBS and antibiotics. Then, the medium was changed every other day, and after 7 days of culture, when the cells reached 80–90% confluency, the cells were passaged or used in the experiments.

### 2.3. Suspension of cells with cryopreservation solution

A commercially available cryopreservation solution, STEM-CELLBANKER GMP grade (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) was used in this study. Cells were detached and dissociated for culture maintenance, described above, and after centrifugation (180g, 3 min), cells were dissociated at a concentration of  $1 \times 10^6$  cells/mL in the cryopreservation solution. The viable cell concentration was determined by an automated cell counter (TC20, Bio-Rad Laboratories, Inc., Hercules, CA, USA) after being stained with trypan blue. The validity of the cell counting measurement is shown in Appendix A of Supplementary material. The hiPSCs and the hMSCs were suspended in the cryopreservation solution with and without  $10 \mu\text{M}$  Y-27632, respectively, for 0–4 h at room temperature, after which the cells were transferred into cryovials (Corning Inc.).

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