

**Points to Consider Regarding Tests to Detect Undifferentiated Pluripotent Stem Cells/Transformed Cells, Tumorigenicity Tests, and Genomic Stability Evaluation for Human Cell-based Therapeutic Products**

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\*This English translation of the Japanese Administrative Notice is intended to be a reference material to provide convenience for users. In the event of inconsistency between the Japanese original and this English translation, the former shall prevail.

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## 1. Introduction

Basic technological requirements for securing the quality and safety of products derived from human cells, among regenerative medical products (referring to “regenerative medical products” as stipulated in Paragraph 9 of Article 2 of the “Act on Securing the Quality, Efficacy, and Safety of Products Including Pharmaceuticals and Medical Devices (Act No. 145 of 1960);” hereinafter, the same shall apply), are stipulated in Notification No. 0208003 issued by Pharmaceutical and Food Safety Bureau (PFSB), the Ministry of Health, Labour and Welfare (MHLW), dated February 8, 2008 [“Guideline on Ensuring the Quality and Safety of Products Derived from the Processing of Autologous Human Cells/Tissues;” hereinafter referred to as “Guideline on autologous human cell-based therapeutic products (CTPs)”] and PFSB Notification No. 0912006 dated September 12, 2008 [“Guideline on Ensuring the Quality and Safety of Products Derived from the Processing of Allogeneic Human Cells/Tissues;” hereinafter referred to as “Guideline on allogeneic human CTPs”]. In addition, securing the quality and safety of products derived from human stem cells, among products derived from human cells, are stipulated in PFSB Notification No. 0907-2 dated September 7, 2012 [“Guideline on Ensuring the Quality and Safety of Pharmaceuticals and Medical Devices from the Processing of Autologous Human Somatic Stem Cells”], PFSB Notification No. 0907-3 dated September 7, 2012 [“Guideline on Ensuring the Quality and Safety of Pharmaceuticals and Medical Devices from the Processing of Allogeneic Human Somatic Stem Cells”], PFSB Notification No. 0907-4 dated September 7, 2012 [“Guideline on Ensuring the Quality and Safety of Pharmaceuticals and Medical Devices from the Processing of Autologous Human Induced Pluripotent Stem(-Like) Cells”], PFSB Notification No. 0907-5 dated September 7, 2012 [“Guideline on Ensuring the Quality and Safety of Pharmaceuticals and Medical Devices from the Processing of Allogeneic Human Induced Pluripotent Stem(-Like) Cells”] and PFSB Notification No. 0907-6 dated September 7, 2012 [“Guideline on Ensuring the Quality and Safety of Pharmaceuticals and Medical Devices from the Processing of Human Embryonic Stem Cells”] (hereinafter referred to as “Five guidelines on human stem cell-based therapeutic products”).

The risk of tumorigenicity associated with transformed cells intermingled with such products is specific to human CTPs. In products derived from human pluripotent stem cells characterized by their capacity for teratoma formation, such as human embryonic stem cell (ESC)-based therapeutic products and human induced pluripotent stem cell (iPSC)-based therapeutic products, there is also the risk of tumorigenicity associated with undifferentiated pluripotent stem cells remaining in the final products. In short, tumorigenic cells, such as transformed cells and pluripotent stem cells, intermingled with human CTPs are hazardous, and it is essential to obtain information on their quantity and types in order to secure the quality and safety of human CTPs. Out of references and points to consider regarding nonclinical evaluation of the quality/safety of human CTPs, this document provides

representative examples of tests that can be used to detect undifferentiated pluripotent stem cells and transformed cells intermingled with human CTPs as well as points to consider in selecting tests from these options to evaluate the quality/safety of specific human CTPs.

## **2. The Role of This Document**

This document focuses on the detection of tumorigenic cells that may be intermingled with a variety of human CTPs, for which technological development is rapidly advancing. The development and technological innovation of the detection tests themselves are also constantly advancing. Under these circumstances, this document provides as many key points as possible at this time, instead of attempting to cover all issues in an exhaustive manner. Therefore, the content of the document is subject to future revision based on further technological innovation and accumulation of knowledge and should not be understood as a binding guideline to follow at the time of application for marketing approval. The document summarizes the characteristics/performance of each test, but does not define which test should be used at which stage of development.

Each product should be flexibly evaluated based on a sufficient understanding of the product in a scientifically rational and adequate manner. Concerning tests and their procedures presented in this document, partial revision or omission will be encouraged if the scientific rationality/validity of such a revision/omission is demonstrated. Furthermore, it is also important to refer not only to this document but also to other relevant guidelines published in Japan and overseas.

## **3. Definition of Terms**

Terms used in this document are as defined below in addition to definitions presented in the Guideline on autologous human CTPs, Guideline on allogeneic human CTPs, Five guidelines on human stem cell-based therapeutic products.

- 1) Tumorigenicity: the capacity of cell populations inoculated into an animal model to produce benign or malignant tumors. This capacity is distinguished from the capacity of chemical or physiologically active substances to immortalize cells to induce benign or malignant tumors (oncogenicity) or only malignant tumors (carcinogenicity). In this document, ESCs/iPSCs (referred to as undifferentiated ESCs/iPSCs when present in products) and transformed cells that may form tumors are handled as tumorigenic cells, irrespective of the presence or absence of evidence from animal studies.

- 2) Cell substrate: the microbial cells or cell lines derived from human or animal sources that possess the full potential for generation of the desired biological products (including regenerative medical products) for human *in vivo* or *ex vivo* use [Notification No. 873 issued by Evaluation and Licensing Division (ELD), Pharmaceutical and Medical Safety Bureau (PMSB), MHLW, dated July 14, 2000, “Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products” (ICH Q5D)].
- 3) Cell bank: a collection of appropriate containers, whose contents are of uniform composition, stored under defined conditions. Each container represents an aliquot of a single pool of cells (“Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products” (ICH Q5D)).
- 4) Raw material: the source of an ingredient or a material used for manufacturing a pharmaceutical, etc. (MHLW Notice No. 375 dated 2014, “Standard for Biological Ingredients”).
- 5) Intermediate product: a material, which is produced in an intermediate manufacturing process and which becomes a product when the remaining part of the process is completed (MHLW Ordinance No. 93 dated 2014, “Ordinance on Standards for Manufacturing Control and Quality Control for Regenerative Medical Products;” hereinafter referred to as GCTP Ministerial Ordinance).
- 6) Tumor: a tissue formed by cells that has grown through excessive autonomous cellular multiplication contrary to the regulatory mechanisms of the body. Tumors are classified into 2 types; benign and malignant tumors.
- 7) Teratoma: a germ cell tumor with diploblastic or triploblastic tissues. Teratomas are classified into 2 groups: mature and immature teratomas.
- 8) Malignant tumor: a tumor that invades surrounding tissues or metastasizes to distant areas of the body.
- 9) Cancer: a malignant tumor in a broad sense, including carcinoma, sarcoma, and hematological malignancy such as leukemia. Carcinoma is a malignant epithelial tumor. Sarcoma is a malignant tumor derived from non-epithelial connective tissues, muscle, or endothelial cells, etc.
- 10) Mass: a lump or swelling found in the body or on the body surface regardless of its cause of occurrence. Concerning the difference between a tumor and a mass, while a mass is a morphological condition of the body including an abscess, a tumor is a pathological condition of the body whereby cells grow.
- 11) Transformed cells: cells that have undergone transformation related to tumorigenicity, such as immortalization and malignant transformation.
- 12) Product cells: cells contained in the product.
- 13) Final product cells: cells contained in the final product.
- 14) Hazard: the potential source of harm. Harm means damage to health [FSB/ELD Notification No.

0901004 and PFSB/Compliance and Narcotics Division (CND) Notification No. 0901005 dated September 1, 2006 “Guideline on Quality Risk Management” (ICH Q9\*)].

- 15) Risk: the combination of the probability of occurrence of harm and the severity of that harm. Severity means a measure of the possible consequences of a hazard (“Guideline on Quality Risk Management” (ICH Q9\*)).

#### **4. General Points to Consider**

There is a wide variety of starting materials for human CTPs such as somatic cells, somatic stem cells, ESCs, and iPSCs. Furthermore, such starting materials can be derived from various types of cells such as autologous cells, allogeneic cells, and HLA homozygous allogeneic cells. Structures and formulations (e.g., cell suspension, cell sheet) of human CTPs are also diverse. The number of cells needed for such a product to be used in clinical practice differs from product to product. In addition, there are many variations in terms of the route of administration, administration site, use status of immunosuppressants, level of patient urgency, and other relevant matters. Therefore, the risk of tumorigenicity in individual product should be evaluated and managed in a comprehensive manner based on scientific understanding of the performance and limitations of each testing method.

#### **5. Tumorigenicity-related Tests for Human ESC/iPSC-based Therapeutic Products**

Tumorigenicity-related tests in manufacturing human ESC/iPSC-based therapeutic products are classified into the following three types depending on testing purpose: (1) tumorigenicity studies of ingredients/raw materials for quality characterization/control; (2) tests to evaluate tumorigenic cells intermingled with the intermediate or the final product; and (3) tests for evaluating the tumorigenic potential of the final product cells at the site of engraftment for clinical application. Tests (1), (2), and (3) are described in Sections 5.1, 5.2, and 5.3, respectively. Table 1 and Table 2 show some examples of *in vitro* and *in vivo* tests for (2). In evaluating tumorigenicity, it is important to understand the principle of each test and confirm its performance including limit of detection, and then to select appropriate test methods and design an evaluation system that is suitable for testing purpose.

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\* ICH Q9 (R1) has been published as of August 31, 2023.

### 5.1. Tumorigenicity tests of ingredients/raw materials for quality characterization/control

In establishing a cell bank for human ESCs or iPSCs as cell substrates used for manufacturing human ESC/iPSC-based therapeutic products, teratoma-forming tumorigenicity may be confirmed to examine cell proliferation and pluripotency of a cell bank. Tumorigenicity studies for this purpose can be performed by reference to Annex 3 of the Technical Report Series No. 978 issued by the 61st meeting of the World Health Organization (WHO) Expert Committee on Biological Standardization in 2013 (WHO TRS 978).<sup>1</sup>

### 5.2. Tests to evaluate tumorigenic cells intermingled with the intermediate or the final product

Besides target cells and their precursor cells, undifferentiated human ESCs/iPSCs and other non-target cells may be intermingled with the cell populations of the intermediates or final products of human ESC/iPSC-based therapeutic products. Given that human ESCs/iPSCs have innately teratoma-forming tumorigenicity, it is necessary to evaluate and manage the quantity of undifferentiated human ESCs/iPSCs intermingled with the intermediate or the final product as well as the quantity of transformed cells, non-target cells that may form tumors, as quality attributes (tumorigenic cells intermingled with the product).

#### 5.2.1. Tests to detect undifferentiated pluripotent stem cells in the intermediate/final product

##### 5.2.1.1. *In vitro* tests

Undifferentiated human ESCs/iPSCs intermingled with the intermediate/final product can be evaluated by detecting molecular markers for human ESCs/iPSCs. Test methods include flow cytometry, quantitative reverse transcription (RT)-polymerase chain reaction (PCR),<sup>2,3</sup> detection of H3+podocalyxin in the culture supernatant,<sup>4</sup> and direct culture amplification of human pluripotent stem cells with laminin 521.<sup>5</sup> However, the validity of the molecular markers to be used should be confirmed in advance for each target cell type for testing. For example, while *LIN28*, of which expression is not found in normal human differentiated cells or human tissues, is considered to be an excellent marker for human ESCs/iPSCs,<sup>2,3</sup> its specificity as an iPSC marker is known to be rather low for the induction of differentiation of human iPSCs into mesenchymal stem cells.<sup>5</sup> Furthermore, undifferentiated human ESCs/iPSCs intermingled with the intermediate/final product cannot be detected by the soft agar colony formation assay, due to apoptosis induced by single cell dispersion with human ESCs/iPSCs.<sup>2</sup>

##### 5.2.1.2. *In vivo* tests

It is recommended that undifferentiated human ESCs/iPSCs intermingled with the product are examined and evaluated by *in vitro* tests using appropriate cell characteristic markers. However, it is also possible, though not essential, to evaluate the quantity of undifferentiated human ESCs/iPSCs

intermingled with the product using tumorigenicity in immunodeficient animals as indicators. Immunodeficient animals include severely immunodeficient mouse strains such as NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Sug/Jic</sup> (NOG) mice<sup>6</sup> and NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl/SzJ</sup> (NSG) mice.<sup>7</sup> These mice lack T cells, B cells, and NK cells and have superior engraftment capacity to human cells and tissues compared to nude mice.<sup>8,9,10</sup> C.Cg-Rag2<sup>tm1Fwa</sup> Il2rg<sup>tm1Sug/Jic</sup> (BRG) mice, a strain lacking T cells, B cells, and NK cells, are known to have a lower engraftment capacity to human hematopoietic stem cells compared to NOG mice or NSG mice.<sup>11,12</sup> Use of SCID mice or NOD/SCID mice is not recommended for tumorigenicity studies requiring prolonged observation because of thymoma that often occurs spontaneously in these mice. The method using nude mice, which is recommended in the WHO TRS 978, is insufficiently sensitive to detect a small number of undifferentiated human ESCs/iPSCs remaining in the product, making it liable to give a false-negative result, and is therefore unsuitable for the evaluation of undifferentiated human ESCs/iPSCs intermingled with the product.<sup>10</sup> In general, a subcutaneous injection into the back skin is given. This approach requires no difficult techniques and allows researchers to transplant many product cells and to observe the time course of tumor formation easily while preventing the occurrence of variability in the results associated with variability in the degree of injection skill. For observing the site of administration, see Section 5.3.6.

If an *in vivo* test is conducted only to quantify undifferentiated human ESCs/iPSCs intermingled with the product cells, which is regarded as exceptional, it is necessary to examine the positive-control cells implanted under an equivalent condition (e.g., an injection site, a manner) at the time of testing the product cells or during preliminary examination. In a test for detecting undifferentiated human ESC/iPSCs remaining in the product, in general, undifferentiated human ESCs/iPSCs, which are cell substrates for manufacturing the product and which may be intermingled with the product cells, are used as the positive-control cells. When administering the product cells, suspending the cells in Matrigel is a more effective way of increasing detection sensitivity.<sup>8,10,13,14</sup> Because human ESCs/iPSCs can undergo apoptosis due to dispersion into single cells by trypsin, etc., it is necessary to take measures to prevent the occurrence of apoptosis when administering such cells to mice. Methods to prevent dispersion-induced apoptosis include the administration of the product without conducting trypsin treatment and the administration of the product cells, which are dispersed by trypsin treatment and are then suspended in Matrigel with a ROCK inhibitor and human neonate-derived fibroblastic cells.<sup>15,16</sup> In product cell transplantation, it is recommended to transplant as many product cells as possible within an artifact-free range taking account of the cell dose needed in actual clinical practice. It is preferable to use 10 or more animals that are eligible for final evaluation in each group when tumor formation is detected. It is thought that any variability in the test population will be generally reflected in the variability of data for quantitative measurement items if evaluation is conducted in at least 6 animals per group.

A test is conducted on the assumption that undifferentiated human ESCs/iPSCs similar to the

positive-control cells remain in the product. Concerning a culture medium needed for the administration of the product cells, therefore, it is recommended to use a medium that is suitable for the proliferation of the positive-control cells, if available, rather than the medium used for administration to humans.

In order to evaluate the quantity of undifferentiated human ESCs/iPSCs intermingled with the intermediate or the final product, it is essential to have a group for the positive-control (the product cells or the human diploid cells used as the negative-control cells, which are spiked with undifferentiated human ESCs/iPSCs). The necessity of having a negative-control group should be determined in consideration of the availability and usability of human diploid cells that may be used as the negative-control cells. The positive-control group to be examined before or at the time of the test should consist of multiple groups transplanted with cellular samples containing undifferentiated human ESCs/iPSCs at various doses, and the minimum tumor-producing dose (TPD<sub>min</sub>) and the time to tumor formation should be confirmed in advance. The animals should be observed until a time point that is sufficiently later than the time point at which the tumor formation rate at TPD<sub>min</sub> of the positive-control group becomes stable. If the objective of the test is to determine the presence of undifferentiated human ESCs/iPSCs intermingled with the product, it is acceptable to use animals of a single sex to enhance the accuracy of the testing system.

The probability that the result obtained from the investigational product is false-negative should be calculated based on the tumor formation rate at TPD<sub>min</sub>. It is also known how to determine the number of animals needed to demonstrate the statistical validity of the result where the quantity of undifferentiated human ESCs/iPSCs intermingled with the investigational product is not higher than the TPD<sub>min</sub>.<sup>8</sup> For other technical details, see Section 5.3.6.

In general, it is practical to conduct an *in vivo* test to detect undifferentiated ESCs/iPSCs that also serves as a test to detect transformed cells in the intermediate or the final product (Section 5.2.2.2).

## 5.2.2. Tests to detect transformed cells in the intermediate/final product

### 5.2.2.1. *In vitro* tests

Transformed cells intermingled with the intermediate or the final product can be evaluated by detecting immortalized and transformed cells through characterization of proliferation of cells cultured for longer than the specified period<sup>17,18</sup> and anchorage-independent proliferating cells (malignant transformed cells) by the soft agar colony formation assay<sup>2,8</sup> or the digital soft agar colony formation assay.<sup>19</sup> However, when using the product in clinical practice, risk assessment should be conducted in consideration of the possible presence of transformed cells that are difficult to detect in any *in vitro* tests.

Characterization of cell proliferation for the detection of immortalized cells is a test that enables cells immortalized during transformation to be detected regardless of the level of malignancy.

Meanwhile, the digital soft agar colony formation assay is a test that enables transformed cells to be detected at high sensitivity. However, this assay cannot detect any cells other than those with anchorage-independent proliferation potential; that is, highly malignant transformed cells. In order to evaluate the quantity of transformed cells intermingled with the intermediate or the final product, tumorigenic transformed cells should be specified as positive-control cells. In conducting a test, it is essential to have a group for the positive control (the product cells or the human diploid cells used as the negative-control cells, which are spiked with the positive-control cells). The quantity of transformed cells intermingled with the investigational product can be determined on the assumption that the tumorigenicity of transformed cells that can be intermingled with the product is equivalent to that of the positive-control cells. Cells whose tumorigenic potential has been sufficiently studied and recognized, such as HeLa cells, should be used as the positive-control cells unless the characteristics of transformed cells that can be intermingled with the product are predicted in advance and a cell line with a similar phenotype is available. For the positive-control cells, it is recommended to use a cell line obtained from a cell provision organization conducting appropriate quality control.

If product cells cannot be cultured under the culture conditions specified for these existing *in vitro* tests and if it is possible to identify genes or molecules characteristic of transformed cells intermingled with the intermediate or the final product, a testing system that enables molecular biological quantification without cultivation may be selected after conducting various validation studies. However, due to such case-by-case tests, general technical requirements or standardized methods cannot be indicated.

#### 5.2.2.2. *In vivo* tests

It is recommended that tumorigenic transformed cells intermingled with the product are examined and evaluated by *in vitro* tests using appropriate cell characteristic markers. However, it is also possible, though not essential, to determine the quantity of transformed cells intermingled with the product using tumorigenicity in immunodeficient animals as an indicator. Immunodeficient animals include severely immunodeficient mouse strains such as NOG mice<sup>6</sup> and NSG mice.<sup>7</sup> These mice lack T cells, B cells, and NK cells and have superior engraftment capacity to human cells and tissues compared to nude mice.<sup>8,9,10</sup> When administering the product cells dispersed by trypsin treatment, suspending the product cells in Matrigel is a more effective way of increasing detection sensitivity.<sup>8,10,13,14</sup> BRG mice, a strain lacking T cells, B cells, and NK cells, are known to have lower engraftment capacity to human hematopoietic stem cells compared to NOG mice or NSG mice.<sup>11,12</sup> Use of SCID mice or NOD/SCID mice is not recommended for tumorigenic tests requiring prolonged observation because of thymoma that often occurs spontaneously in these mice. The method using nude mice, which is recommended in the WHO TRS 978, is insufficiently sensitive to detect a small number of tumorigenic transformed cells remaining in the product, making it liable to give a false-negative result, and is therefore

unsuitable for the purpose presented in this section.<sup>8,10</sup>

In order to evaluate the quantity of transformed cells intermingled with the intermediate or the final product, it is necessary to specify the tumorigenic transformed cells that will serve as the positive-control cells. To be more specific, it is necessary to have a group for the positive control (the product cells or the human diploid cells used as the negative-control cells, which are spiked with the positive-control cells) and to examine its tumorigenicity at the time of testing the product cells or during a preliminary test. The positive-control group should consist of multiple groups transplanted with cellular samples containing the positive-control cells at various doses, and the TPD<sub>min</sub> and the time to tumor formation should be confirmed in advance. Tests designed as described above enable transformed cells intermingled with the product cells to be quantitatively evaluated.

Cells whose tumorigenic potential has been sufficiently studied and recognized, such as HeLa cells, should be used as positive-control cells unless the characteristics of transformed cells that can be intermingled with the product are predicted in advance and cell lines with similar phenotypes are available. The positive-control group spiked with the positive-control cells at various doses should be established in advance, and the TPD<sub>min</sub> of the positive-control cells should be evaluated in advance. It should be noted that the quantity of transformed cells intermingled with the investigational product to be calculated is determined on the assumption that the tumorigenicity of transformed cells that can be intermingled with the product is equivalent to that of the positive-control cells. For the positive-control cells, it is recommended to use a cell line obtained from a cell provision organization conducting appropriate quality control.

In general, a subcutaneous injection into the back skin is given as this approach requires no difficult techniques and allows researchers to observe the time course of tumor formation easily. It also prevents variability in the results that be a consequence of variability in the degree of injection skill. However, in cases where particularly high attention needs to be paid to the relationships between tumorigenicity and clinical route/site of transplantation (see Section 5.3), the injection site should be the same as the one used in clinical practice as far as possible. For observing the site of administration, see Section 5.3.6.

At the time of testing or during preliminary examination, it is necessary to confirm the TPD<sub>min</sub> for the positive-control group in advance and to observe animals until a time point that is sufficiently later than the time point at which the tumor formation rate at TPD<sub>min</sub> of the positive-control group becomes stable. It is also useful to calculate the dose at which a tumor formed in 50% of animals transplanted with the positive-control cells [tumor producing dose at the 50% end-point (TPD<sub>50</sub>)].<sup>1</sup> TPD<sub>50</sub> is a quantitative index for the tumorigenicity of the positive-control cells and can be used as the target value for comparison when discussing the tumorigenicity of transformed cells intermingled with product cells. The number of test animals should be determined in consideration of relevant factors such as the difficulty of transplantation and the survival ratio of test animals. Although it is preferable

to have 10 or more animals that are eligible for final evaluation in each group, it is thought that any variability in the test population will generally be reflected in the variability of data for quantitative measurement items if evaluation is conducted in at least 6 animals that are eligible for final evaluation in each group.

When using a medium that is suitable for the proliferation or survival of the product cells as a medium to be administered together with the product cells, it is necessary to confirm that the tumorigenicity of the positive-control cells is also maintained in the medium or that the proliferation of the positive-control cells is not inhibited in the medium. When using a medium that is suitable for the proliferation of the positive-control cells, it should be noted that the medium may not be suitable for the survival or proliferation of some of the transformed cells that may be intermingled with the product cells.

In product cell transplantation, it is recommended to transplant as many product cells as possible into each animal within an artifact-free range, taking account of the cell dose needed in actual clinical practice. When no tumors are observed, the probability of the result obtained from the investigational product being false-negative should be calculated based on the tumor formation rate of the positive-control cells at  $TPD_{min}$ . It is also known how to determine the number of animals needed to demonstrate the statistical validity of the result where the quantity of transformed cells intermingled with the investigational product is not higher than the  $TPD_{min}$ .<sup>8</sup> For other technical details, see Section 5.3. If the objective of the test is to evaluate the presence of tumorigenic transformed cells intermingled with the product, it is acceptable to use animals of a single sex to enhance the accuracy of the testing system. The necessity of having a negative-control group should be determined in consideration of the availability and usability of human diploid cells that may be used as the negative-control cells.

It should be noted that this test is conducted to determine if tumorigenic cells are present (or not) in the intermediate or the final product, not to directly evaluate tumorigenicity in humans. The examples of tests evaluated in the strictest manner are shown above. It is recommended that when planning any *in vivo* test, the results of *in vitro* tests need to be taken fully into consideration. For example, in cases where an *in vivo* test is conducted simply to confirm the results of an *in vitro* study, the content and stringency of the *in vivo* test may be modified according to the objectives of the test, taking the detection sensitivity and accuracy of the *in vitro* test into account. The microenvironment of the transplantation site is known to affect the tumorigenicity of transformed cells.<sup>20,21</sup> Therefore, in a test of subcutaneous transplantation into the back skin, for example, a risk assessment of the product should be conducted while remaining aware of the possibility that transformed cells that do not form any tumors when transplanted subcutaneously into the back skin, may do so in cases where the product is administered by a method other than subcutaneous transplantation. Information obtained from the literature on studies of transplantation of specific human cancer cell types into animals [patient-derived xenograft (PDX)], if available, should also be used as reference.

### 5.3. Tests to evaluate the tumorigenic potential of the final product cells at the site of engraftment for clinical application

The major information needed for the evaluation of the tumorigenicity of the final product is as follows: (1) quantity of undifferentiated human ESCs/iPSCs intermingled with the product; (2) quantity of transformed cells intermingled with the product; and (3) tumorigenic potential of cells administered at the site of engraftment. Concerning (1) and (2), evaluation can be conducted through the detection of molecular markers for pluripotent stem cells, and the detection of immortalized cells or anchorage-independent proliferating cells, respectively. The *in vivo* tests shown in Sections 5.2.1.2 and 5.2.2.2 are also usable for evaluation. Concerning (3), however, there are no methods to evaluate the tumorigenic potential of administered cells at the site of engraftment other than *in vivo* tumorigenicity studies. In conducting such studies, consideration should be given to the following: (a) selection of test animals; (b) control cell selection, detection power of the testing system; (c) number of test animals; (d) administration site of the test sample, number of cells in the sample, and the form of the sample; (e) duration of observation; (f) observation of the administration site; (g) histological evaluation of the administration site, identification of human cells administered and the confirmation of engraftment, histological evaluation of the degree of differentiation; and (h) interpretation of results. Regarding the administration site, a site equivalent to the administration site in humans should be selected as far as possible. This is because tumorigenic potential or tumor type may vary depending on the microenvironment of the engraftment site,<sup>20,21</sup> which may become a problem when trying to extrapolate study results to humans. If there are limitations in terms of the number of cells to be administered to the administration site due to various reasons such as physical obstruction, it is recommended to adjust the number of cells administered at the administration site in proportion to the relative scale ratio of animals and humans instead of changing the site, if possible. In other words, priority should be given to investigating the possibility of tumor formation due to interactions between the engraftment microenvironment and cells administered. *In vivo* tests in animals are thought to probably provide significant information on cellular behavior in unusual administration environments associated with such factors as immune privilege, inflammation, and ischemia. However, elements such as humoral factors and receptor proteins forming the human body microenvironment show high specificity to humans. Therefore, when considering the extrapolation of the test results to humans, it should be also noted that how much animal models can reflect the microenvironment of the engraftment site in human bodies is unclear at the preclinical stage.

#### 5.3.1. Selection of test animals

Immunodeficient animals used for the stable engraftment of human CTPs include severely immunodeficient mouse strains such as NOG mice<sup>6</sup> and NSG mice.<sup>7</sup> These mice lack T cells, B cells, and NK cells and have superior engraftment capacity to human cells and tissues compared to nude

mice.<sup>8,9,10</sup> BRG mice, a strain lacking T cells, B cells, and NK cells, are known to have lower engraftment capacity to human hematopoietic stem cells compared to NOG mice or NSG mice.<sup>11</sup> Therefore, when selecting animals for a test, it is necessary to take the objectives of the test into consideration.

Use of SCID mice or NOD/SCID mice lacking T cells and B cells is not recommended because of thymoma which often occurs spontaneously in these mice and may affect the interpretation of test results. Spontaneous tumors rarely occur in NOG mice aged less than 48 weeks.<sup>22</sup> Use of disease model animals may also be considered in cases where there are problems with cellular behavior in unusual administration environments associated with such factors as immune privilege, inflammation, and ischemia. In such cases, it is necessary to investigate in advance the degree to which the disease model animal represents the pathological characteristics of the target disease. However, although a testing system using a disease model animal whose validity has been demonstrated is suitable for evaluating efficacy, it may not be suitable for evaluation in tumorigenicity studies which require long-term administration of immunosuppressants and for which a stable, long-term testing system that allows consistent statistical conclusions to be made is needed. Therefore, use of animal disease models should be determined in consideration of study objectives and other relevant factors. In fact, for the reasons given above, immunodeficient animals, into which human cells can be easily transplanted, are often used in tumorigenicity studies, instead of using disease model animals. Although no animal species, in which the immune system is suppressed as much as it is in NOG and NSG mice, are available other than mice, mice have some disadvantages such as the small size of the administration site and difficulty in producing disease models. In some tests, therefore, immunodeficient animals that are larger than mice, such as nude rats lacking T cells, are used.<sup>10,14,23</sup> For animals that are even larger than rats, immunosuppressants may need to be concomitantly administered because of the difficulty in obtaining sufficiently immune-suppressed animals. These animals may be usable in tests to obtain data supporting the efficacy and performance of the product within a short period, but not in tests requiring a prolonged period of time such as tumorigenicity studies.

### 5.3.2. Selection of control cells

In *in vivo* tumorigenicity studies with immunodeficient animals, it is recommended to have the positive-control group using the product cells or the negative-control human diploid cells spiked with the positive-control cells. The type of positive-control cell varies depending on the tumorigenic cells expected to be intermingled with the product. If the characteristics of tumorigenic cells intermingled with the product can be predicted in advance and a cell line with a similar phenotype is available, such a cell line should be selected. If such a cell line is not available, cells whose tumorigenic potential has been sufficiently studied and recognized, such as HeLa cells, should be used as the positive-control cells. For the positive-control cells, it is recommended to use a cell line obtained from a cell provision

organization conducting appropriate quality control. If a tumorigenic intermediate product is used for the positive control group, it is necessary to examine the quantity of tumorigenic cells in the intermediate product by a separate *in vitro* test or other relevant tests. If it proves to be difficult to have a positive-control group, the meaningfulness of conducting tumorigenicity study must be reviewed taking into account the fact that it would be hard to ascertain whether a negative test result is actually true or false without a positive-control group. This leads to necessity of explaining that expected performance of a test is obtained and what can be evaluated from the test results. The necessity of having a negative-control group should be determined in consideration of the availability and usability of human diploid cells that can be used as the negative control cells.

In cases where no undifferentiated ESCs/iPSCs are intermingled with the product, while the characteristics of the transformed cell expected to be intermingled with the product are clarified and appropriate transformed cells used as the positive control are available, it is important to specify  $TPD_{50}$ ,  $TPD_{min}$  and time for tumor detection with a test evaluating the transformed cells at the site of engraftment and to transplant the product cells into the site of engraftment. However, such cases are considered to be rare. When the characteristics of tumorigenic cells intermingled with the product have not been clarified in advance, it should be noted that the results of a test should be interpreted on the assumption that the tumorigenicity of tumorigenic cells intermingled with the product is equivalent to that of the positive-control cells.

### 5.3.3. Number and sex of test animals

It is preferable to have 10 or more animals that are eligible for final evaluation in each group in consideration of the difficulty of transplantation and the survival ratio of test animals. It is thought that any variability in the test population will generally be reflected in the variability of data for quantitative measurement items if evaluation is conducted in at least 6 animals per group. It is also known the number of animals is determined by the statistical validity of the result where the quantity of undifferentiated human ESCs/iPSCs intermingled with the investigational product is not higher than the  $TPD_{min}$  of the positive-control group.<sup>8</sup> In tests to evaluate the tumorigenic potential of the final product cells in humans at the site of engraftment, approximately the same number of male and female animals may be used in one group if effects of sex are observed. However, it is acceptable to use animals of a single sex if effects of sex difference on capability of tumor formation can be ignored (e.g., clinical application on a single sex).

### 5.3.4. Administration sites of the test sample, number of cells in the sample, and the form of the sample

Generally, doses of low molecular weight drugs in nonclinical safety studies using animal models are determined by considering inter-species and inter-individual difference. These doses should be more than doses applied in clinical studies based on uncertainty factors (safety factors). However, in

*in vivo* tumorigenicity studies of human CTPs, it is often difficult to administer the same number of cells to test animals as to humans or more because of the limited size of the animals. If there are limitations on the number of cells that can be administered to animals at the same administration site by the same route of administration as that in humans due to various reasons such as physical obstruction, it is recommended to adjust the number of cells administered at the administration site in proportion to the relative scale ratio of animals and humans instead of changing the site, if possible. In other words, priority should be given to investigating the possibility of tumor formation due to interactions between the engraftment microenvironment and cells administered. Selection of a relative scale ratio, which includes area ratio and volume ratio, should be determined for each product based on the formulation (dosage form) of the product and the characteristics of its usage. Priority should be given to the administration site because cellular behavior in unusual administration environments associated with factors such as immune privilege, inflammation, and ischemia is difficult to evaluate without using *in vivo* tests in model animals. However, this shall not apply if the use of other administration sites can be rationalized as in the following cases: 1) there is a technical difficulty in administering to a site corresponding to the administration site in humans; 2) there is a difficulty in interpreting the results of a test; and 3) it is obvious that administration to other sites is superior in terms of test performance including skills and sensitivity. The form of the sample to be administered should be the same as the formulation (dosage form) of the final product, if possible.

#### 5.3.5. Duration of observation

Concerning all animals in each group, it is recommended to observe physical condition every day, measure body weight and examine for the presence or absence of any tumor formed at the site of administration of the product cells by direct observation, palpation, and diagnostic imaging at least once a week. However, actual observation frequency and examination items should be determined with consideration of the potential burden on animals such as administration sites, anesthesia and time needed for imaging. Duration of observation varies depending on presence or absence of a positive-control group and a predictive lifetime of transplanted cells (and their dividing cells) *in vivo*. In the case where there is a positive-control group [the final product (or the negative-control cells similar to the normal cells of the same cell type) spiked with the positive-control cells],  $TPD_{min}$  for the positive-control group should be confirmed at the time of the test or during the preliminary examination. The presence or absence of tumorigenicity can be determined by observing animals until a time point that is sufficiently later than the time point at which the tumor formation rate at  $TPD_{min}$  becomes stable. In such a case, the presence or absence of tumorigenicity can be determined on the assumptions that the tumorigenicity of tumorigenic cells that can be intermingled with the product is equivalent to that of the positive-control cells and that the  $TPD_{min}$  is the minimum dose needed for tumor formation. In subcutaneous administration to NOG mice, while tumor formation rates in human

iPSCs and HeLa cells are known to become nearly stable within 4 to 6 months,<sup>8,10,13,14</sup> the test facility should confirm the time needed for tumor formation rate at  $TPD_{min}$  to become stable because this period can vary depending on skill, cell handling, or culture history.

Even with a positive-control group, a longer observation period may be needed if the transformation of cells administered is likely to result in tumor formation at the engraftment site in cases such as where the genomic stability of cells in the final product is apparently low. However, there are limits to how long the observation period can be extended for due to various factors including animal life span.<sup>24</sup> Therefore, even if tumor formation is not found during a prolonged observation period, it is important to take advance precautionary measures in the clinical setting against the possibility of tumor formation due to cellular transformation after administration, such as follow-up and a risk management plan with surgical resection and drug therapy in advance.

When testing in the absence of a positive-control group, it is recommended to monitor animals for tumor formation over the period from administration of the final product cells to the death of animals, the longest period in which evaluation is free from any impact of naturally occurring disease, or the period until transplanted cells (and their dividing cells) are no longer identifiable.

A rationally justifiable observation period should be specified in consideration of not only the presence or absence of a positive-control group but also other relevant factors including animal species, strain, pathology, and immunocompromised status.

#### *5.3.6. Observation of administration sites*

If the formation of a mass is detected at the administration site, the date of detection should be documented. It is recommended to evaluate the growth of the mass by measuring its minor and major axes at least once a week if it is possible to confirm the formation of a mass by appearance such as a subcutaneous site. However, actual measurement should be planned and implemented taking account of the potential burden on animals such as anesthesia and measurement time.

In the case of excessive growth of the mass, animals should be sacrificed by the pre-specified method in terms of animal welfare. If regression of the mass is observed, animals should not be sacrificed before the end of the pre-specified observation period. If there is no observable growth of a mass, the mass should not be immediately regarded as a tumor.

#### *5.3.7. Pathological evaluation*

All animals should be sacrificed at the end of the observation period, and necropsy should be performed to examine the administration site and formed masses. In necropsy, grossly distinguished mass tissues as well as major organs with abundant blood flow (e.g., liver, spleen, kidney, lung, lymph nodes) should be isolated, and mass tissues should be examined by hematoxylin-eosin (HE) staining or immunostaining to clarify what type of tumor tissues human-derived cells has formed. Other

isolated organs should be visually inspected for the presence or absence of a mass. It is also useful to perform evaluation by immunostaining with anti-human HLA antibodies or Alu PCR<sup>25,26</sup> with a focus on the human cellular gene-specific Alu sequence to examine the status of invasion/distant metastasis of human cells. The performance (e.g., sensitivity, specificity) of these evaluation methods should be examined in advance. All the isolated tissues should be stored. Even if no mass is detected during the observation period, the cell product transplanted in animals should be isolated and the histology of the transplanted human cells should be studied to prevent false-negative results. In this case, it is essential to demonstrate the following based on the results of histopathological examination and appropriate immunostaining: a) transplanted cells survived until the isolation of the graft, b) transplanted cells had no tumorigenic potential, and c) the observed histology of transplant was suitable for the purpose of product usage. Also similarly to the earlier-stated cases, major organs with abundant blood flow (e.g., liver, spleen, kidney, lung, lymph nodes) should be isolated and stored for future analysis.

#### 5.3.8. Interpretation of results

If tumor formation is observed in the product cell group, it is necessary to clarify whether or not the cells comprising the tumor are derived from human cells and to consider modifying the manufacturing method or quality specifications of the product while examining the quantity of tumorigenic cells intermingled with the product. If tumor formation is not observed in the product cell group, the possibility of false-negative results should be investigated based on the performance of an *in vivo* tumorigenicity study, which is suggested by the TPD<sub>min</sub> for the positive-control cells.<sup>8</sup> Tumorigenic transformed cells such as cancer cells are highly diverse and may include cell types that are rarely detected in *in vivo* tumorigenicity studies. Therefore, information obtained from the literature on studies of implanting particular human cancer cell types into animals (patient-derived xenograft; PDX), if available, may also be used as reference. The interpretation of the test results should be based on the assumption that the tumorigenicity of tumorigenic cells intermingled with product cells is equivalent to that of positive control cells.

## 6. Tumorigenicity-related Tests for Human Somatic Cell-/Somatic Stem Cell-based Therapeutic Products

### 6.1. Tumorigenicity tests of ingredients/raw materials for quality characterization/control

Annex 3 to Technical Report Series No. 978 issued by the 61st meeting of the WHO Expert Committee on Biological Standardization in 2013 (WHO TRS 978)<sup>1</sup> should be used as reference when tumorigenicity tests are conducted to characterize the quality of a cell bank of human somatic cells or human somatic stem cells established for use as cell substrates (ingredients or raw materials) for

manufacturing human somatic cell-/somatic stem cell-based therapeutic products.

## 6.2. Points to consider regarding tumorigenicity-related tests for the final product

With respect to tumorigenicity of human somatic cell-/somatic stem cell-based therapeutic products as the final products, it is necessary to examine the quantity of transformed cells intermingled with the product and the tumorigenic potential of administered cells at the site of engraftment based on study data or information from the literature.

Human cell transplantation and the clinical application of human somatic cell- or human somatic stem cell-based therapeutic products have been advancing worldwide. However, there have been only a limited number of reports on cases where tumors formed due to such cell transplantation or administration of CTPs including the following: a report on a benign tumor formed in the brain after treatment of ataxia telangiectasia with a human fetal neural stem cell-based product;<sup>27</sup> a case of the formation of a mass after autologous olfactory mucosal grafting (no cell processing) for treating spinal cord injury;<sup>28</sup> and a case of tumor formation in the spinal cord of a patient with ischemic stroke receiving intraspinal administration of mesenchymal stem cells, ES cells, and fetal neural stem cells at a clinic as part of so-called “stem cell tourism.”<sup>29</sup> There have also been extremely rare cases of leukemia caused by the donor’s cells after allogeneic hematopoietic stem cell transplantation despite the fact that an increase in the risk of hematologic tumor was not observed in the donor.<sup>30</sup> Concerning human mesenchymal stem cell-based therapeutic products that are widely used in regenerative medicine or cell therapy, no cases of tumor formation have been reported after single-agent administration in clinical practice. While four cases of malignant transformation of human mesenchymal stem cells have been reported during *in vitro* cultivation, two of these cases were found later to be caused by the cross contamination with a cancer cell line.<sup>31,32</sup> In the other two cases, immortalization of cells was confirmed during *in vitro* cultivation.<sup>33,34</sup> As suggested in these cases, it is important to prevent cross contamination with tumorigenic cells of the final product and to understand cell proliferation profiles. In general, there is no need to perform any *in vivo* tumorigenicity study using immunodeficient animals as a nonclinical safety study in relation to human somatic cell-/mesenchymal stem cell-based therapeutic products that have been cultured and processed in accordance with GCTP Ministerial Ordinance, and in which the absence of abnormalities has been confirmed by characterization of the proliferation of cells cultured for a duration that exceeds the specified culture period.

However, tumorigenicity tests with immunodeficient animals designed as shown in Section 5.3 should be considered in addition to tests such as characterization of proliferation of cells cultured for a duration that exceeds the specified culture period in the following cases. (1) Tumors are likely to be formed at the site of engraftment after administration for reasons including the following: cells in the final product are highly proliferative; the degree of differentiation of cells in the final product is poor;

and the final product contains cells that are similar to reported tumorigenic cells. (2) The final product is non-homologous used. (3) Administration of a product manufactured from the same cell lines (products derived from human allogeneic cells) to many patients possibly leads to spreading the risk of tumorigenicity.

## **7. General Points to Consider Regarding Genomic Stability**

Reduced genomic stability is a potential hazard related to the risk of tumorigenicity because it is expected to increase the probability of karyotypic abnormalities or genomic mutations and to thereby increase the probability of cellular transformation.

Genomic mutations, such as karyotypic changes, are known to occur in human cells when cultured. Even in human diploid fibroblast cells with a stable karyotype, minor mutations are detected by a single nucleotide polymorphism (SNP) array-based analysis. In addition, non-diploid karyotypes are sometimes observed even in apparently normal tissues. The safety of cells with genomic mutations such as karyotypic abnormalities, which are seen *in vitro*, has not been concluded. Genomic information that serves as the baseline for genomic stability differs depending on cell type or culture method. No cells exhibit absolute stability of genomic replication in passage culture. Therefore, it is necessary to limit the length of the culture period and the number of passages to minimize genomic instability, a hazard, and to perform risk assessment of culture conditions and the effect of changes in the culture conditions.

Tests for genomic stability include G-banded karyotyping, fluorescence in situ hybridization (FISH), array CGH, SNP array, and next-generation sequencing. Changes in chromosome number, translocation, and other reconfiguration data of a cell can be confirmed by G-banded karyotyping. This method is useful for demonstrating a normal diploid karyotype for a certain number of passages or divisions, which can be used as a rough indicator for genomic stability. Array CGH has the advantage of enabling a change in copy number in a smaller gene region to be detected. With respect to FISH and next-generation sequencing, it is important to evaluate the appropriateness of using these as testing methods by verifying the relationship between the information obtained by these methods and tumorigenicity in a scientific manner while studying their detection sensitivity to genomic mutation (mutation types and allele frequencies) and the availability of adequate controls. If its performance can be verified, a simple chromosomal test by a next-generation sequencer (digital karyotyping) may replace G-banded karyotyping, which is time-consuming and inadequate in terms of quantification performance.

These tests are generally helpful in characterizing the product cells. At present, however, they are performed to obtain reference information of cell characteristics rather than being used as a

specification to determine whether the product can be released.

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**Table 1 Methods to Detect/Quantify Undifferentiated ESCs/iPSCs Intermingled with Product Cells**

Test method	<i>In vivo</i> tumorigenicity test (subcutaneous administration with Matrigel in NOG mice)	Flow cytometry	qRT-PCR
Objective	Detection of tumorigenic cells	Detection of undifferentiated pluripotent stem cells	Detection of undifferentiated pluripotent stem cells
Duration of test/analysis (italic)	17 – 30 weeks	<i>1 day</i>	<i>Approximately 6 hours</i>
Advantages	<ul style="list-style-type: none"> <li>◆ Direct testing</li> <li>◆ Able to evaluate tumorigenicity within the microenvironment corresponding to the site of transplantation in actual clinical practice</li> </ul>	<ul style="list-style-type: none"> <li>◆ Quick</li> <li>◆ Able to analyze individual cells and evaluate the expression of marker molecules</li> <li>◆ Easy to perform</li> </ul>	<ul style="list-style-type: none"> <li>◆ Rapid</li> <li>◆ High sensitivity</li> <li>◆ Easy to perform</li> </ul>
Disadvantages; Points to consider	<ul style="list-style-type: none"> <li>◆ Costly and time-consuming</li> <li>◆ Requires dedicated facilities for animals</li> <li>◆ Low throughput</li> <li>◆ Requires pathological analysis, etc. to clarify whether tumors found are derived from transformed cells or</li> </ul>	<ul style="list-style-type: none"> <li>◆ Indirect testing</li> <li>◆ Gating affects the results</li> </ul>	<ul style="list-style-type: none"> <li>◆ Indirect testing</li> <li>◆ Unable to evaluate the expression of marker molecules in individual cells</li> </ul>

	pluripotent stem cells		
Detection performance or detection limit (underlined)	Detects human iPSCs, which are intermingled with hRPE at a ratio of 1,000 in $2.5 \times 10^5$ (0.4%), with a probability of 50%	<u>0.1% of human iPSCs in hRPE (marker: TRA-1-60)</u>	<u>≤ 0.002% of human iPSCs in hRPE (marker: LIN28)</u>
Source	Kanemura <i>et al.</i> , <i>Sci Rep.</i> 2013 Kawamata <i>et al.</i> , <i>J Clin Med.</i> 2015	Kuroda <i>et al.</i> , <i>PLoS ONE.</i> 2012	Kuroda <i>et al.</i> , <i>PLoS ONE.</i> 2012

**Table 1 (continued) Methods to Detect/Quantify Undifferentiated ESCs/iPSCs Intermingled with Product Cells**

Test method	Droplet digital PCR	GlycoStem-HP	Essential-8/LN521 culture amplification
Objective	Detection of undifferentiated pluripotent stem cells	Detection of undifferentiated pluripotent stem cells	Detection of undifferentiated pluripotent stem cells
Duration of test/analysis (italic)	<i>Approximately 6 hours</i>	$\leq 3$ hours <i>(from collection of culture supernatant to measurement)</i>	Approximately 1 week
Advantages	<ul style="list-style-type: none"> <li>◆ Rapid</li> <li>◆ Easy to perform</li> <li>◆ High sensitivity</li> </ul>	<ul style="list-style-type: none"> <li>◆ Does not destroy cells</li> <li>◆ Easy to perform</li> <li>◆ High throughput</li> </ul>	<ul style="list-style-type: none"> <li>◆ Direct</li> <li>◆ Easy to perform</li> <li>◆ Able to characterize residual iPSCs</li> </ul>
Disadvantages; Points to consider	<ul style="list-style-type: none"> <li>◆ Indirect testing</li> <li>◆ Unable to evaluate the expression of marker molecules in individual cells</li> </ul>	<ul style="list-style-type: none"> <li>◆ Indirect testing</li> <li>◆ Unable to evaluate the expression of marker molecules in individual cells</li> <li>◆ Medium components affect the results</li> </ul>	<ul style="list-style-type: none"> <li>◆ Time-consuming</li> <li>◆ Low throughput</li> </ul>
Detection performance or detection limit (underlined)	<ul style="list-style-type: none"> <li>◆ <u>0.001% of human iPSCs in human cardiac myocytes (marker: <i>LIN28</i>)</u></li> </ul>	<ul style="list-style-type: none"> <li>◆ 0.05% of human iPSCs in HEK293T (marker: H3 + podocalyxin)</li> </ul>	<ul style="list-style-type: none"> <li>◆ 0.01% to 0.001% of human iPSCs in hMSC</li> <li>◆ 0.1% to 0.01% of human iPSCs in human embryoid</li> </ul>

Source	Kuroda <i>et al.</i> , <i>Regen Ther.</i> 2015	Tateno <i>et al.</i> , <i>Sci Rep.</i> 2014	Tano <i>et al.</i> , <i>PLoS ONE.</i> 2014
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**Table 2 Methods to Detect/Quantify Transformed Cells Intermingled with Product Cells**

Test method	<i>In vivo</i> tumorigenicity study (subcutaneous administration with Matrigel in NOG mice)	Soft agar colony formation assay	Digital soft agar colony formation assay	Characterization of cell proliferation
Objective	Detection of tumorigenic cells	Detection of anchorage-independent growth (malignant transformed cells)	Detection of anchorage-independent growth (malignant transformed cells)	Detection of immortalized cells (transformed cells)
Duration of test	≥ 16 weeks	3 – 4 weeks	3 – 4 weeks	≥ 4 weeks
Advantages	<ul style="list-style-type: none"> <li>◆ Direct testing</li> <li>◆ High sensitivity</li> <li>◆ Able to evaluate tumorigenicity within the microenvironment corresponding to the site of transplantation in actual clinical practice</li> </ul>	<ul style="list-style-type: none"> <li>◆ Inexpensive</li> <li>◆ Able to isolate/characterize malignant transformed cells</li> </ul>	<ul style="list-style-type: none"> <li>◆ High sensitivity</li> <li>◆ Able to isolate/characterize malignant transformed cells</li> </ul>	<ul style="list-style-type: none"> <li>◆ Inexpensive</li> <li>◆ Easy to perform</li> <li>◆ Able to detect a wide range of immortalized cells besides malignant transformed cells</li> </ul>
Disadvantages; Points to consider	<ul style="list-style-type: none"> <li>◆ Costly and time-consuming</li> <li>◆ Requires dedicated facilities for animals</li> <li>◆ Requires pathological analysis, etc. to clarify whether tumors found are derived from transformed</li> </ul>	<ul style="list-style-type: none"> <li>◆ Indirectly determines the presence or absence of tumorigenic cells</li> <li>◆ Not usable for planktonic cells</li> <li>◆ Unable to detect immortalized cells besides</li> </ul>	<ul style="list-style-type: none"> <li>◆ Indirectly determines the presence or absence of tumorigenic cells</li> <li>◆ Not usable for planktonic cells</li> <li>◆ The image scanner is expensive</li> </ul>	<ul style="list-style-type: none"> <li>◆ Indirectly determines the presence or absence of tumorigenic cells</li> <li>◆ Unable to determine the presence or absence of malignant transformed cells</li> </ul>

	<p>cells or pluripotent stem cells</p> <ul style="list-style-type: none"> <li>◆ Unable to detect immortalized cells not showing tumorigenicity <i>in vivo</i></li> </ul>	<p>malignant transformed cells</p>	<ul style="list-style-type: none"> <li>◆ Unable to detect immortalized cells besides malignant transformed cells</li> </ul>	
<p>Detection performance or detection limit (underlined)</p>	<p>Able to detect HeLa cells, which are intermingled with hMSC at a ratio of 1/10<sup>6</sup> (0.0001%; 10 cells), with a probability of 17%</p>	<p>HeLa cells, which are intermingled with hMSC at a ratio of 1/10<sup>3</sup> (0.1%) (calculated detection limit: 0.02%)</p>	<p>HeLa cells, which are intermingled with hMSC at a ratio of 1/10<sup>7</sup> (0.00001%)</p>	<p>HeLa cells, which are intermingled with hMSC at a ratio of 1/10<sup>6</sup> (0.0001%), and immortalized adipose-derived stem cells, which are intermingled with adipose-derived stem cells at a ratio of 1/10<sup>5</sup> (0.001%)</p>
<p>Source</p>	<p>Kusakawa et al., <i>Regen Ther.</i> 2015</p>	<p>Kusakawa et al., <i>Regen Ther.</i> 2015</p>	<p>Kusakawa et al., <i>Sci Rep.</i> 2015</p>	<p>Kono et al., <i>Biologicals.</i> 2015&amp;2017 Hasebe-Takada et al., <i>Regen Ther.</i> 2016</p>

## **Reference Information 1: *In vivo* tumorigenicity study to detect undifferentiated ESCs/iPSCs intermingled with product cells**

Source: Kanemura *et al.* Tumorigenicity studies of induced pluripotent stem cell (iPSC)-derived retinal pigment epithelium (RPE) for the treatment of age-related macular degeneration.

*PLoS ONE*. 2014;9:e85336.

### **Methodology**

#### *1. Cell Culture*

The human iPS cell line 201B7, which was created from skin fibroblasts transduced with retroviruses, pMXs-POU5F1, -Sox2, -c-Myc, and -Klf4, are maintained on the SNL feeder cell layer using a ReproFF2 medium containing 5 ng/mL bFGF. The cell line 836B1 was established from skin fibroblasts collected from normal adults. The cell lines 59, K11, K21, 101, RNT9, and RNT10, are derived from the skin fibroblasts of 6 patients with retinitis pigmentosa associated with photoreceptor-specific gene mutations, from whom informed consent was obtained. From skin fibroblasts, iPSCs were created using an EBNA-based episomal vector into which POU5F1, SOX2, KLF4, MYCL, LIN28A, and GLIS1 (iPS cell lines 59-G, K21-G, 101-G, RNT9, and RNT10) or POU5F1, SOX2, KLF4, MYCL, LIN28A, and p53shRNA (iPS cell lines 101-EV, K11-EV, and K21-EV) were inserted. These iPSCs are maintained on autologous fibroblast-derived feeder cells using a primate ES medium containing 5 ng/mL bFGF. iPS cell-derived retinal pigment epithelium (RPE) cell clones (59-G3 RPE, K21-G18 RPE, 101-G25 RPE, RNT9 RPE, RNT10 RPE, 101-EV RPE, and K11-EV9 RPE or K21-EV15 RPE) are maintained in an RPE culture medium [Dulbecco's Modified Eagle's Medium:Ham's F12 Medium (7:3) containing B-27 supplement, 2 mM L-glutamine, 0.5 nM SB431542, and 10 ng/mL bFGF]. Human primary RPE cultures are maintained in the retinal pigment epithelial cell basal medium containing L-glutamine, GA-1000, and bFGF. Suspension-cultured human iPS cell-derived RPE cells are used for subcutaneous administration or the production of RPE cell sheets applied onto the collagen gel to link with collagen. The RPE cell sheet is maintained in the Ham's F10 medium with 10% fetal bovine serum (FBS) for 4 weeks and in an RPE maintenance medium for 3 weeks and then is detached from the collagen gel with collagenase I. The RPE cell sheet is subcutaneously administered with a mixture of suspended cells and Matrigel, or cut into pieces by the laser microdissection to be used for retinal implant in animals.

#### *2. Animal Experiments*

##### *2.1. Subcutaneous transplantation in mice*

Inject various doses of Hela cells, which are mixed with 200  $\mu$ L of Matrigel or suspended in 200  $\mu$ L of PBS (without Matrigel), into the subcutaneous tissue of female nude mice (BALB/cAJCl-*nu/nu*), SCID mice (C.B-17/*Icr-scid/scidJcl*), NOD-SCID mice (NOD/ShiJic-*scidJcl*), or NOG mice

(NOD/ShiJic-*scid*, IL-2R $\gamma$ KO Jic) aged 7 to 8 weeks using a 1 mL syringe with a 26G needle. Monitor the animals for 36 weeks. Sacrifice the mice at the end of the experiment, isolate tumors, and fix them with 4% paraformaldehyde. Stain the paraffin sections with hematoxylin-eosin (HE) for pathological observation. Inject various doses of the human iPSCs 201B7 or RPE cells derived from  $1 \times 10^6$  human iPSCs, which are mixed with 200  $\mu$ L of Matrigel or suspended in 200  $\mu$ L of PBS (without Matrigel), subcutaneously into female NOG mice aged 7 to 8 weeks using a 1 mL syringe with a 26G needle. Monitor the mice for 6 to 15 months. Sacrifice the mice at the end of the experiment and isolate grafts with tweezers. Fix the grafts with 4% paraformaldehyde.

## 2.2. Subretinal administration to rats

Anesthetize female nude rats (F344/NJcl-*rnu/rnu*) aged 3 weeks by intraperitoneal administration of a mixture of ketamine 100 mg/kg and xylazine 10 mg/kg. Dilate the right pupil with mydriatic drops (0.5% tropicamide, 0.5% phenylephrine hydrochloride). Make a small incision in the sclera in the outer corner of the right eye with a 27G needle. Inject various doses of HeLa cells, human iPSCs, or a 1 mm square of human iPSC cell-derived RPE cell sheet soaked in 2  $\mu$ L of DMEM/F12 medium from the incision site on the sclera into the subretinal space using a Hamilton syringe with a 33G needle. Transplant the cells or the RPE cell sheet onto the subretinal capillary plexus while tracking the location of the Hamilton syringe through the dilated pupil under a surgical microscope. Use the subretinal capillary plexus, which can be easily observed in albino nude rats, as an indicator of the location of the subretinal space. Monitor the nude rats transplanted with the cells/cell sheet for 8 to 82 weeks. Sacrifice the rats at the end of the experiment. Isolate eyeballs transplanted with the cells/cell sheet and fix them with 4% paraformaldehyde.

## 3. RT-PCR and Quantitative RT-PCR

Extract total RNA using a kit and remove any genomic DNA contamination by spin column centrifugation. Create cDNA from 50 ng total RNA using PrimeScript RT Master Mix and PrimeSTAR MAX DNA Polymerase. Quantitative PCR should be performed using SYBR Green, and the gene expression level should be corrected based on glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Quantitative RT-PCR should be performed using QuantiTect Probe RT-PCR Kit. Expression levels of the target genes should be corrected based on RNase P transcription. A total of 45 cycles of quantitative RT-PCR should be performed. The sequences of the probes and primers that are used in this experiment are presented in “Table: Sequences of Probes and Primers (Reference Information 1).”

## 4. Alu PCR

Use human cell-specific Alu sequences for designing primers. Use Alu primer, 5'-

AAGTCGCGGCCGCTTGCAGTGAGCCGAGAT-3', 50 ng DNA template, and PrimeSTAR Max DNA Polymerase for PCR reaction (28 cycles). Use DNA templates for various ratios of human HeLa cell DNA versus mouse NIH3T3 DNA to determine the detection sensitivity of Alu PCR. Separate PCR products by electrophoresis with 1% agarose gel and digitally scan the images.

##### *5. Immunohistochemistry*

Fix the transplanted tissues with 4% paraformaldehyde. Stain paraffin-embedded tissue sections with hematoxylin-eosin (HE). After HE staining, treat the paraffin-embedded sections first with xylene and then sequentially with 100%, 95%, 80%, and 70% ethanol for 5 minutes each for the respective levels for deparaffinization. Treat the sections with 10 mM citric acid (pH 6) at 95°C for 50 minutes and then with 0.4% Triton-X100/PBS at room temperature for 30 minutes. Stain the deparaffinized sections with anti-human Lamin-A antibody, anti-BEST1 antibody, and anti-Ki-67 antibody. Stain the nuclei with Hoechst 33258 or DAPI. Fix human iPS cell-derived RPE cells in suspension with 4% paraformaldehyde and stain with anti-POU5F1 (OCT3/4) antibody or anti-BEST1 antibody. Visualize antibodies using Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 488 goat anti-rabbit IgG. Scan fluorescence micrographs using a fluorescence microscope.

**Table: Sequences of Probes and Primers (Reference Information 1)**

Primers for RT-PCR			
Gene	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')	
<i>LIN28A</i>	CACGGTGCGGGCA TCTG	CCTTCCATGTGCAG CTTACTC	
<i>POU5F1</i>	GAAACCCACACTG CAGCAGA	TCGCTTGCCCTTCT GGCG	
<i>BEST1</i>	ATCAGAGGCCAGG CTACTACAG	TCCACAGTTTTCCT CCTCACTT	
<i>CRALBP</i>	GACTGGGGTTAAA TCTCACAGC	TGACATGTTGCCTA TGGAAGAC	
<i>PAX6</i>	TTAACACACTTGAG CCATCACC	AAATCTCGGATGTC TGTCCACT	
<i>TYR</i>	AGCCAGCATCATT CTTCTC	GGCGTTCATTGCA TAAAGA	
<i>GAPDH</i>	CGATGCTGGCGCT GAGTAC	CCACCACTGACAC GTTGGC	
Probes and primers for qRT-PCR			
Gene	Probe sequence (5' – 3')	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')
<i>LIN28A</i>	CGCATGGGGTTCG GCTTCCTGTCC	CACGGTGCGGGCA TCTG	CCTTCCATGTGCAG CTTACTC
<i>POU5F1</i>	CGGACCACATCCTT CTCGAGCCCAAGC	GAAACCCACACTG CAGCAGA	TCGCTTGCCCTTCT GGCG

**Reference Information 2: qRT-PCR as a method to detect undifferentiated ESCs/iPSCs intermingled with product cells**

Source: Kuroda *et al.* Highly sensitive in vitro methods for detection of residual undifferentiated cells in retinal pigment epithelial cells derived from human iPSCs.

*PLoS ONE.* 2012;7(5):e37342

**Methodology**

*1. Total RNA Extraction*

Extract total RNA from sample cells (e.g., differentiated iPSCs) in accordance with the protocol provided with the kit and treat it with deoxyribonuclease (DNase).

*2. Quantitative RT-PCR*

2.1. Prepare PCR mixtures as follows, using the QuantiTect Probe RT-PCR Kit.

a) PCR mixture (*LIN28*)

	Final conc.	Assay/well (μL)
QuantiTect RT Mix	1 ×	0.25
2 × QuantiTect Probe RT-PCR Master Mix	1 ×	12.5
100 μM Forward Primer	0.4 μM	0.1
100 μM Reverse Primer	0.4 μM	0.1
20 μM Probe	0.1 μM	0.125
RNase free water	-	6.93
		Total 20

b) PCR mixture (*GAPDH*)

	Final conc.	Assay/well (μL)
QuantiTect RT Mix	1 ×	0.25
2 × QuantiTect Probe RT-PCR Master Mix	1 ×	12.5
10 μM Forward Primer	0.2 μM	0.5
10 μM Reverse Primer	0.2 μM	0.5
5 μM Probe	0.1 μM	0.5
RNase free water	-	5.75
		Total 20

Use TaqMan® GAPDH Control Reagents (human).

2.2 Prepare total RNA solutions as follows:

a) For measurement of *LIN28*

- Preparation of calibration curve templates

Dilute with RNase free water so that the concentration of undifferentiated iPS cell-derived RNA becomes 0.1, 0.03, 0.01, 0.003, 0.001, and 0 ng/ $\mu$ L, respectively.

- Preparation of sample RNA

Prepare sample RNA so that its concentration becomes 10 ng/ $\mu$ L.

b) For measurement of *GAPDH*

- Preparation of calibration curve templates

Dilute with RNase free water so that the concentration of undifferentiated iPS cell-derived RNA becomes 10, 3, 1, 0.3, 0.01, and 0 ng/ $\mu$ L, respectively.

- Preparation of sample RNA

Dilute with RNase free water so that the concentration of sample RNA becomes 1 ng/ $\mu$ L.

2.3. Place a 20  $\mu$ L aliquot of the PCR mixture in each well of a 96-well plate for PCR.

2.4. Add the template solution at 5  $\mu$ L per well (and mix well).

2.5. Place the well plate into the real-time PCR system.

Conditions for Quantitative RT-PCR

Stage	Temperature	Time
Stage 1	50.0°C	30 minutes
Stage 2	95.0°C	15 minutes
Stage 3	94.0°C	15 seconds
	60.0°C	1 minute
Repeat "Stage 3" 45 cycles (40 cycles for <i>GAPDH</i> )		

Plate Layout (for Samples A, B, C)

	<i>GAPDH</i>			<i>LIN28</i>								
	1	2	3	4	5	6	7	8	9	10	11	12
A	201B7	201B7	201B7	201B7*	201B7*	201B7*						
B	A	A	A	A	A	A						
C	B	B	B	B	B	B						
D	C	C	C	C	C	C						
E	S10	S3	S1	S3	S1	S0.3						
F	S0.3	S0.1	DW	S0.1	S0.03	S0.01						
G				S0.003	S0.001	DW						
H												

\*201B7 for the measurement of undifferentiated markers should be prepared to obtain a concentration of 1 ng/ $\mu$ L (positive control).

Sequences of *LIN28* Probes and Primers

Gene	Probe Primer set (5' – 3')	
<i>LIN28</i>	Probe sequences (5' FAM/3' TAMRA)	CGCATGGGGTTCGGCTTCCTGTCC
	Forward primer sequences	CACGGTGCGGGCATCTG
	Reverse primer sequences	CCTTCATGTGCAGCTTACTC

The primer and the probe should be prepared to obtain concentrations of 100  $\mu$ M and 20  $\mu$ M, respectively.

**Note:** As an assessment criterion for a trace of *LIN28* detected, classify any Ct value exceeding 35 as “not detected.” (Substantial variability is expected when Ct is higher than 35.)

### Reference Information 3: Droplet digital PCR as a method to detect undifferentiated ESCs/iPSCs intermingled with product cells

Source: Kuroda *et al.* Highly sensitive droplet digital PCR method for detection of residual undifferentiated cells in cardiomyocytes derived from human pluripotent stem cells.

*Regen Ther.* 2015;2:17-23.

#### Methodology

##### 1. Total RNA Extraction

Extract total RNA from sample cells (e.g., differentiated iPSCs) in accordance with the protocol provided with the kit and treat it with DNase.

##### 2. Droplet Digital PCR

2.1. Prepare the PCR mixture as follows, using One-Step RT-ddPCR Kit for Probes.

PCR mixture

	Final conc.	Assay/well ( $\mu\text{L}$ )
2 $\times$ One-Step RT-ddPCR Supermix	1 $\times$	10
25 mM Manganese	1 $\times$	0.8
50 $\mu\text{M}$ Forward Primer	0.75 $\mu\text{M}$	0.3
50 $\mu\text{M}$ Reverse Primer	0.75 $\mu\text{M}$	0.3
50 $\mu\text{M}$ Probe	0.25 $\mu\text{M}$	0.1
RNase free water	-	3.5
		Total 15

2.2. Prepare total RNA solutions as follows:

For measurement of *LIN28*

- Preparation of calibration curve templates  
Dilute with RNase free water so that the concentration of undifferentiated iPS cell-derived RNA becomes 0.1, 0.03, 0.01, 0.003, 0.001, and 0 ng/ $\mu\text{L}$ , respectively.
- Preparation of sample RNA  
Prepare sample RNA so that its concentration becomes 10 ng/ $\mu\text{L}$ .

2.3. Pour the PCR mixture at 15  $\mu\text{L}$ /well into a PCR tube.

2.4. Add the RNA solution at 5  $\mu\text{L}$ /well (and mix well).

2.5. Generate droplets using Droplet Generator.

2.6. Transfer the droplet solution into a 96-well plate.

2.7. RT-PCR reaction

Thermal Cycler Conditions

Stage	Temperature	Time
Stage 1	60.0°C	30 minutes
Stage 2	95.0°C	5 minutes
Stage 3	94.0°C	30 seconds
	64.0°C	1 minute
Repeat "Stage 3" 40 cycles.		
Stage 4	98°C	10 minutes

2.8. Analyze the PCR reaction solution using QX100 Droplet Reader.

Sequences of *LIN28* Probes and Primers

Gene	Sequence (5' – 3')	
<i>LIN28</i>	Probe sequences (5' FAM/3' BHQ1)	CGCATGGGGTTCGGCTTCCTGTCC
	Forward primer sequences	CACGGTGCGGGCATCTG
	Reverse primer sequences	CCTCCATGTGCAGCTTACTC

Both the primers and probe should be prepared to obtain a concentration of 50 µM.

**Notes:**

- Given that the optimal annealing temperature varies from primer to primer, the conditions should be reviewed as needed.
- It should be noted that the result can differ markedly depending on the threshold specified.

#### **Reference Information 4: Non-destructive *in vitro* tumorigenicity study using culture supernatant**

Source: Tateno *et al.* A medium hyperglycosylated podocalyxin enables noninvasive and quantitative detection of tumorigenic human pluripotent stem cells.

*Sci Rep.* 2014;4:4069.

#### **Kits/Instruments Needed for Measurements**

- Human ES/iPS cell monitoring kit
- Centrifuge (capable of centrifugation at  $1,700 \times g$ )
- Test tube mixer
- Plate mixer (if available)
- 96-well plate washer (if available)
- 96-well plate reader (absorbance determination: dominant wavelength, 450 nm; secondary wavelength, 600 to 650 nm)

#### **Notes**

##### *Notes regarding measurements*

- Sample culture supernatant on the day after medium replacement, and detach cells to count the number of human pluripotent stem cells. When the medium is 5 mL and the number of cells is  $5 \times 10^6$  cells, for example, the number of undifferentiated cells in the sampled culture supernatant should be  $1 \times 10^6$  cells/mL.
- In this method, the standard curve should be prepared based on the measurements of the culture supernatant under the conditions of maintenance culture for undifferentiated cells, and the number of undifferentiated cells in the sample for measurement should be calculated based on the standard curve.
- The relationship between the signal intensity and the number of cells (cells/mL) may differ depending on the cell line or culture conditions including the type of medium. A standard curve should be prepared for each cell line and each condition of maintenance culture for undifferentiated cells.

##### *Use of kit*

- Store the kit at a temperature of 20°C to 25°C until use.
- Wash the well plate using a device such as a plate washer, invert the plate, and tap it gently onto paper towels or the like to remove the wash solution remaining in wells.
- Put all the kit components other than the plate seal back into the refrigerator promptly for storage after use.

#### *Sample (culture supernatant) preparation method*

- Collect supernatant following culture for 18 to 24 hours after medium replacement, and use it as samples for measurement and for preparation of standard curves.
  - \*All the medium should be considered to be replaced in principle because the duration of culture time after medium replacement affected measurement. It is recommended to unify the time to sampling after medium replacement so far as possible.
- Centrifuge the sampled culture supernatant at  $1,700 \times g$  (3,000 rpm) for 10 minutes at room temperature. Use the recovered centrifuge supernatant as the sample.
- Cryopreserve the supernatant at  $\leq -20^{\circ}\text{C}$  if it is not immediately used for measurement.

#### *Standard curve preparation*

- Prepare the standard curve for each cell line and for each condition of maintenance culture for undifferentiated cells.
- Collect the culture supernatant 18 to 24 hours after replacement of all the medium, and detach cells to measure the number of undifferentiated cells. Prepare samples from the culture supernatant in accordance with “Sample (culture supernatant) preparation method” shown above. Cryopreserve the samples at  $\leq -20^{\circ}\text{C}$  until they are used for measurement. (The samples can withstand several freezing-and-thawing cycles.)
- Prepare the standard curve by diluting the sample with a fresh medium that is the same as the medium used. It is recommended to dilute the sample to a concentration of 30,000 cells/mL, which is followed by further serial 3-fold dilutions up to 41 cells/mL to examine its dilution profile. Determine the appropriate number of cells and dilute it further by the serial 2-fold dilution method to prepare the calibration curve. Taking account of the potential high background noise from some media, be sure to prepare wells containing only individual media.
- It is recommended to use one of the strips for the standard curve in each measurement, and to calculate the number of undifferentiated cells in the sample for measurement based on the standard curve.
- When it is difficult to use one of the strips for the standard curve in each measurement because of a large number of samples, the number of wells used for standard curves can be reduced (to 2-4 wells). However, be sure to choose the number of cells with which a linear relationship can be obtained.

### **Operation**

#### *Preparation*

- Ensure that the following are at room temperature before use: rBC2LCN solid phase plate,

negative control, positive control, diluent, wash solution (10 ×), stop solution, and plate seal (reagents other than an HRP-labeled antibody solution and a TMB solution).

- Dilute the wash solution (10 ×) 10 times with distilled water at room temperature. At least 40 mL of the wash solution (1 ×) is needed per strip (350 μL/well × 12 washings × 8 wells). Prepare the wash solution according to the number of measurements (number of strips to be used). A larger volume of wash solution should be prepared in consideration of the amount of the liquid needed for setting up apparatus if using a plate washer.
- Dilute the positive control for checking the status of reaction 40 times with the diluent immediately before use (add 5 μL of the positive control to 195 μL of the diluent, mix them with a vortex mixer, and pour into available wells at 50 μL/well).
- The negative control should be placed into wells without dilution.
- Dilute the HRP-labeled antibody solution 20 times with the diluent and prepare the required amount of solution (50 μL/well × the number of wells) immediately before use. The HRP-labeled antibody solution should be returned to the refrigerator promptly after the required amount has been taken out.
- Dispense the required amount of the TMB solution (50 μL/well × the number of wells) into sterilized fresh tubes 20 to 30 minutes before chromogenic reaction and store at room temperature until use, protected from light. The TMB solution should be returned to the refrigerator promptly after the required amount has been taken out.

#### *Measurement procedure*

1. After confirming that the rBC2LCN solid phase plate is at room temperature, take out the plate from the bag. Take off strips that are not used for measurement from the plate frame, and put them back into the bag. Zip up the bag and store it in the refrigerator.
2. Close the plate frame holder to fix strips and wash 3 times with 350 μL/well of the wash solution (× 1). Invert the plate, and tap it gently onto paper towels or the like to remove the wash solution remaining in wells.
3. Into each well, add 50 μL each of the sample for standard curves and the sample for measurement, and, if needed, the negative control and the positive control. After mixing them gently using a plate mixer or other appropriate device, place the plate seal onto the plate and allow to stand for 1 hour at room temperature to react.
4. Take off the plate seal, and wash the plate 3 times with 350 μL/well of the wash solution (× 1). Invert the plate, and tap it gently onto paper towels or the like to remove the wash solution remaining in wells.
5. Add 50 μL of HPR-labeled antibody solution diluted 1:20 into each well, stir gently with a plate mixer, place the plate seal, and allow to stand for 1 hour at room temperature.

6. Remove the plate seal, and wash 6 times with 350  $\mu\text{L}$ /well of the wash solution (1  $\times$ ). Invert the plate, and tap it gently onto paper towels or the like to remove the wash solution remaining in wells.
7. Add 50  $\mu\text{L}$  of TMB solution into each well, stir gently with a plate mixer or the like, and allow to stand for 30 minutes at room temperature to react (protect from light with a sheet of aluminum foil, etc.).
8. Add 50  $\mu\text{L}$  of stop solution to each well, mix gently to stop reaction, and measure the absorbance (dominant wavelength, 450 nm; secondary wavelength, 620 to 650 nm) with a plate reader. Any foams/bubbles, if present, should be pricked with a pipet tip to eliminate before measurement.
9. Calculate the number of undifferentiated cells in the sample for measurement based on the standard curve.  
  
\*Ensure that the absorbance of the positive control, if used, is 0.5 or higher and that the absorbance of the negative control, if used, is less than 0.15.

#### **Operation procedure (flow chart)**

rBC2LCN solid-phase plate

| Wash 3 times.

Into each well, add 50  $\mu\text{L}$  each of the sample for standard curves and the sample for measurement, and, if needed, the negative control and the positive control.

| Agitate and allow to stand for 1 hour to react at room temperature.

| Wash 3 times.

Add 50  $\mu\text{L}$  of diluted HRP-labeled antibody solution (1 in 20) into each well.

| Agitate and allow to stand for 1 hour to react at room temperature.

| Wash 6 times.

Add 50  $\mu\text{L}$  of TMB solution into each well.

| Agitate and allow to stand for 30 minutes to react at room temperature, protected from light.

Add 50  $\mu\text{L}$  of stop solution into each well.

| Agitate.

Determine the absorbance (dominant wavelength: 450 nm; secondary wavelength: 600 to 650 nm).

## **Reference Information 5: Essential 8/LN521 culture-amplification as a method to detect undifferentiated ESCs/iPSCs intermingled with product cells**

Source: Tano *et al.* A novel *in vitro* method for detecting undifferentiated human pluripotent stem cells as impurities in cell therapy products using a highly efficient culture system.

*PLoS ONE*. 2014;9:e110496.

### **Methodology**

#### *1. Detection of residual undifferentiated iPSCs during the process of differentiation of human iPSCs into mesenchymal stem cells*

##### *1.1. Preparation of a laminin-521 (LN521) coating plate*

Add LN521 diluted with PBS to 20 µg/mL into a culture plate (1 mL/10 cm<sup>2</sup>) and incubate for 2 hours or longer at 37°C. Then, remove LN521 and wash it with PBS. After washing with the Essential 8 medium once, add the Essential 8 medium to the culture plate (2 mL/10 cm<sup>2</sup>) and incubate at 37°C until cell seeding.

##### *1.2. Preparation of the positive control*

Disperse human bone marrow-derived mesenchymal stem cells (hMSCs) as differentiated cells in the Essential 8 medium. This medium is spiked with the original iPS cell line used for differentiation induction, which is dispersed in the Essential 8 medium as single cells (for example, if the proportion of iPSCs intermingled with hMSCs is 1%, 0.1%, or 0.01%, spike  $1 \times 10^5$  MSCs with  $1 \times 10^3$ ,  $1 \times 10^2$ , or  $1 \times 10$  iPSCs, respectively. If the proportion of iPSCs intermingled with hMSCs is 0.001%, spike  $6 \times 10^5$  hMSCs with 6 iPSCs). After mixing differentiated cells and human iPSCs well, place the mixture into the LN521 coating plate prepared as instructed in 1.1. and incubate at 37°C with 5% CO<sub>2</sub> [When the number of hMSCs is  $1 \times 10^5$ , use a 35-mm dish (or a 6-well plate); when the number of hMSCs is  $6 \times 10^5$ , use a 100-mm dish]. Replace the medium with fresh medium on the daily basis starting from 2 days after the initiation of culture. The medium is collected with a pipette tip or a pipette without using an aspirator until forming colonies can be visually observed.

##### *1.3. Test sample preparation*

Detach cells that have been induced to differentiate from human iPSCs with Accutase and disperse the cells in the Essential 8 medium. Transfer the cells into the LN521 coating plate prepared as instructed in 1.1 and culture at 37°C with 5% CO<sub>2</sub>. Medium replacement should be performed as instructed in 1.2.

##### *1.4. Detection of residual undifferentiated iPSCs*

Residual iPSCs proliferate within approximately 1 week of the start of the culture and form

colonies. Confirm the presence or absence of colonies and count the number of colonies formed, if applicable. The colonies that are formed is immunostained with antibodies to undifferentiated markers including TRA-1-60 to confirm that they are derived from undifferentiated iPSCs.

It is necessary to examine the detection sensitivity to residual iPSCs using the positive control in order to determine the presence or absence of residual cells. This method enables the approximate residue level to be estimated by comparing with the number of colonies detected in the positive control.

## **Reference Information 6: *In vivo* tumorigenicity study as a method to detect transformed cells intermingled with product cells**

Source: Kusakawa *et al.* Characterization of *in vivo* tumorigenicity tests using severe immunodeficient NOD/Shi-*scid* IL2R $\gamma$ null mice for detection of tumorigenic cellular impurities in human cell-processed therapeutic products.

*Regen Ther.* 2015;1:30-37.

### **Methodology**

#### *1. Tumorigenicity Tests with NOG Mice*

##### *1.1 Cell culture*

As transplantation cells, use HeLa cells (transformed cells) and human bone marrow-derived mesenchymal stem cells (hMSCs; normal cells). With respect to culture media, use Eagle's minimum essential medium containing 10% FBS (10%FBS/MEM) for the culture of HeLa cells and mesenchymal stem cell growth medium (MSCGM) for the culture of hMSCs.

##### *1.2 Observation of cell transplantation and mass formation*

1.2.1. From a flask where 80% of its surface is covered with cells, detach individual cells with 0.25% trypsin-EDTA solution. Prepare cell suspensions with the following concentrations: 1) to  $10^6$  hMSCs, add  $10^1$  (0.001%),  $10^2$  (0.01%),  $10^3$  (0.1%), and  $10^4$  (1%) HeLa cells, respectively; and 2) to  $10^7$  hMSCs, add  $10^1$  (0.0001%),  $10^2$  (0.001%), and  $10^4$  (0.1%) HeLa cells, respectively. In a mixture of 10% FBS/MEM and Matrigel (1:1), prepare 100  $\mu$ L each of cell suspensions for transplantation that contain the above numbers of cells. Place the suspensions on ice until just before transplantation.

1.2.2. Transplant 100  $\mu$ L of the suspension subcutaneously into the back skin of male NOG mice (NOD/Shi-*scid* IL2R $\gamma$ KO Jic) aged 6 to 8 weeks using a 1-mL syringe with a 25G needle. Use at least 6 mice per group.

1.2.3. Examine to ascertain the presence or absence of a mass formed by visual inspection and palpation every week for 16 weeks. If any mass is found, measure its minor and major axes with a caliper. Obtain the mass volume ( $\text{mm}^3$ ) using the following formula: major axis (mm)  $\times$  minor axis<sup>2</sup> ( $\text{mm}^2$ )  $\times$  1/2. When the mass weight (calculate from the mass volume assuming that the specific gravity is 1) exceeds more than 10% of the body weight or when 16 weeks of observation is completed, sacrifice all the animals and perform necropsy. Preserve all the mass tissues isolated for pathological evaluation in a 10% neutral buffered formalin solution.

1.2.4. Obtain the frequency of mass formation (the number of mice in which mass formation is

detected/the number of mice transplanted) for each cell level group, and calculate the 50% tumor producing dose (TPD<sub>50</sub>) based on the Spearman-Kärber method.

### *1.3 Evaluation of the presence/absence of tumorigenic cells intermingled with normal cells*

From the results of the positive control cells, the probability of non-occurrence of mass formation in a mouse [false-negative rate (x) = 1 – frequency of mass formation] can be obtained. The formula for calculating the probability of non-detection of mass formation (y) in (n) mice transplanted is expressed as  $y = x^n$ . Based on these formulas, the following formula can be obtained:  $n = \log y / \log x$ . Using this formula, it is possible to calculate the number of animals needed for a study according to an acceptable false-negative rate. For example, if the mass formation rate following the transplantation of  $10^7$  hMSCs intermingled with 10 HeLa cells (percentage of HeLa cells intermingled with hMSCs: 0.0001%) is 17%, the probability of non-occurrence of mass formation [false-negative rate (x)] in a mouse transplanted with cells containing tumorigenic cells equivalent to HeLa cells at a ratio of  $1/10^6$  is 0.83. Assuming that the acceptable probability of false-negative results is 1%, no mass formation should be detected in any mice following the transplantation of  $10^7$  cells to each of 25 mice ( $= \log 0.01 / \log 0.83$ ) in order to demonstrate that tumorigenic cells equivalent to HeLa cells are not intermingled with normal cells at a ratio of  $1/10^6$ .

## Reference Information 7: Digital soft agar colony formation assay as a method to detect transformed cells intermingled with product cells

Source: Kusakawa *et al.* Ultra-sensitive detection of tumorigenic cellular impurities in human cell-processed therapeutic products by digital analysis of soft agar colony formation.

*Sci Rep.* 2015;5:17892

### Methodology

#### 1. Cell Culture and Reagents

Use HeLa cells as transformed cells, and human bone marrow-derived mesenchymal stem cells (hMSCs) as normal cells. With respect to culture media, use 10% FBS/MEM and mesenchymal stem cell growth medium (MSCGM) for the usual culture of HeLa cells and hMSCs, respectively. As soft agar media, use 10% FBS-containing 1 × Dulbecco's modified Eagle's medium (DMEM), 20% FBS-containing 2 × DMEM prepared with powder DMEM (phenol red-free medium), and 1.2% agarose solution prepared with low-melting agarose (SeaPlaque) and sterile water. As fluorescent reagents for staining viable cells, use MitoTracker Red CMXRos and Hoechst 33342. As the 96-well plate, it is recommended to use a plate with a plastic bottom that is not treated for cell culture and, if possible, with black side-walls suitable for image analysis. Use a Terasaki plate, 0.25% trypsin-EDTA solution, 4% PFA solution, PBS, Buffer QG (buffer for dissolving agar medium), and the High Content Imaging System.

#### 2. Testing Methods

##### 2.1 Soft agar colony formation assay

Perform three-dimensional (3D) cell culture using the medium composition shown below.

Medium layer 100 $\mu$ L (10% FBS-containing 1 × DMEM)
Cell/soft agar layer 75 $\mu$ L (10% FBS-containing 1 × DMEM and 0.4% agarose)
Bottom layer of agar medium 50 $\mu$ L (10% FBS-containing 1 × DMEM and 0.6% agarose)

Figure: Soft Agar Culture System (Cross-section view of a well of a 96-well plate)

Prior to culture, warm 10% FBS-containing 1 × DMEM and 20% FBS-containing 2 × DMEM to 37°C. Dissolve 1.2% agarose solution with a microwave oven and maintain its temperature at 37°C.

Preparation of the bottom layer of the agar medium: Mix 20% FBS-containing  $2 \times$  DMEM and 1.2% agarose solution at a ratio of 1:1, and dispense a 50  $\mu$ L aliquot of the mixture into each well of a 96-well plate. Transfer the plate into the refrigerator (4°C) and leave it there for 30 minutes to solidify. Preparation of the cell/soft agar layer: Detach cells with 0.25% trypsin-EDTA solution and prepare them at various concentrations using 10% FBS-containing  $1 \times$  DMEM (for example, prepare a suspension with a concentration of  $4 \times 10^5$  cells/mL when seeding  $10^4$  cells per well,  $10^4$  cells/25  $\mu$ L). Mix a cell suspension prepared with 10% FBS-containing  $1 \times$  DMEM, 20% FBS-containing  $2 \times$  DMEM, and 1.2% agarose solution at a ratio of 1:1:1, dispense a 75  $\mu$ L aliquot of the mixture onto the solidified bottom layer of agar medium in a 96-well plate. Transfer the plate into the refrigerator (4°C) and leave it there for 15 minutes.

Medium layer: Add 100  $\mu$ L of 10% FBS-containing  $1 \times$  DMEM onto the cell/soft agar layer. Replace the medium with fresh medium every 3 to 4 days, and culture in an incubator at 37°C with 5% CO<sub>2</sub> for 30 days.

## *2.2 Image analysis of colonies using the High Content Imaging System*

Staining: After 30 days of culturing, remove 100  $\mu$ L of the medium from each well with a pipet, add 25  $\mu$ L each of 10% FBS-containing  $1 \times$  DMEM that contains viable cell-staining reagents (6  $\mu$ g/mL Hoechst 33342, 150 nM MitoTracker Red CMXRos) (the final concentration of Hoechst 33342, 1  $\mu$ g/mL; the final concentration of MitoTracker Red CMXRos, 25 nM), and culture in an incubator at 37°C with 5% CO<sub>2</sub> for 1 hour. Solidification: Remove 10% FBS-containing  $1 \times$  DMEM that contains viable cell-staining reagents with a pipet (after adding 100  $\mu$ L of PBS, remove both the DMEM and PBS), add 125  $\mu$ L of 4% PFA solution to each well (the final concentration of PFA, 2%), and allow to stand for 30 minutes at room temperature. Dissolution and sedimentation treatment: Remove PFA, wash twice with PBS (add 100  $\mu$ L, allow to stand for 10 minutes, and remove PBS), add 50  $\mu$ L of Buffer QG into each well, and incubate at 37°C for 1 hour. The nuclei of colonies formed and mitochondria are stained blue and red, respectively, in the course of the above treatment, and the colonies settle out onto the bottom of the well.

Store the plate in the refrigerator until image analysis (add PBS to each well to prevent evaporation of the medium).

## *2.3 Obtaining images*

Obtain images from 4 visual field locations for each well of the 96-well plate using a 4 times objective lens. Images should be obtained by each of 3 channels (blue, red, and bright field).

## *2.4 Image analysis*

Put together the images of each well taken from 4 visual field locations to obtain the complete

image of each well. Extract recognized regions from each of the blue and red fluorescence images using the pre-designed image analysis script (evaluation indices: size, circularity, and fluorescence intensity), and consider that a colony is present when the extracted regions overlap each other. Visually inspect colonies in the bright field images to confirm that they do not reflect non-specific staining of debris and the like.

### *2.5 Confirmation of detection sensitivity in the positive control cells (detection of a single HeLa cell intermingled with $10^7$ hMSCs)*

Preparation of HeLa cells: Prepare a HeLa cell suspension containing 50 to 100 HeLa cells/mL, and dispense a 10  $\mu$ L aliquot of the suspension into each well of a Terasaki plate. Examine the wells in which single HeLa cells are present under the microscope. Preparation of hMSCs: Prepare a cell suspension containing  $10^7$  hMSCs and mix it with 1.2% agarose solution and 20% FBS-containing  $2 \times$  DMEM in a reservoir (e.g., 4.4 mL of hMSC suspension with a concentration of  $2.5 \times 10^6$  cells/mL + 4.4 mL of 1.2% agarose solution + 4.4 mL of 20% FBS-containing  $2 \times$  DMEM). Add single HeLa cells isolated from the Terasaki Plate with a pipet, and place an aliquot of it in each of 160 wells (80 of the 96 wells of each of two 96-well plates) using a multi-channel pipet. In this method, 75  $\mu$ L of the cell/soft agar layer contains 62,500 MSCs and 0.00625 HeLa cells; that is, one of the 160 wells contains one single HeLa cell.

Perform soft agar culture and image analysis using the above method. In addition, confirm that no colonies are detected in the negative control by also performing soft agar culture of only hMSCs without HeLa cells.

### *3. Ascertaining the Presence/Absence of Malignant Transformed Cells Intermingled with Normal Cells*

Based on the results of the positive control cells, ascertain if malignant transformed cells corresponding to the positive control cells are present or not among normal cells. If HeLa cells are used as the positive control cells, it is the presence or absence of cells corresponding to HeLa cells that should be ascertained. From the results of the positive control cells, the probability of non-detection of colonies in a divided well of the sample [= the number of wells without colonies/the number of divisions (probability distribution of the number of wells without colonies)] can be obtained. In each assay (division of the sample into multiple wells), the probability of non-detection of colonies ( $x$ ) (= the probability of non-detection of colonies in a divided well of the sample<sup>n</sup>) can be obtained. The formula for calculating the probability of non-detection of colonies (false-negative rate) ( $y$ ) in all the ( $n$ ) assays is expressed as  $y = x^n$ . Using this formula, it is possible to calculate the number of assays needed to achieve an acceptable false-negative rate. In other words, it is possible to estimate the number of assays needed to rule out the presence of malignant transformed

cells intermingled with the investigational cellular sample based on the results of the positive control cells. An example is presented below.

Assume that the results shown in the following table are obtained for the positive control cells; that is, a cellular sample consisting of  $10^7$  hMSCs and one HeLa cell.

The number of colonies in a well	Soft agar colony formation assays were performed with a cellular sample consisting of $10^7$ hMSCs and one HeLa cell, which was divided into 160 wells (6 assays in total)							
	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Mean	Probability distribution of the number of wells
0	159	160	159	159	160	159	159.3	0.9956
1	1	0	1	1	0	1	0.7	0.0044

The probability of non-detection of colonies in a well; that is, the probability distribution of the number of wells with no colonies (the number of wells with no colonies/the number of divisions =  $159.3/160$ ) is 0.9956. The probability of non-detection of colonies in one assay (the number of divisions of the sample into multiple wells = 160) (x); that is, the probability of non-detection of colonies in a divided well of the sample<sup>x</sup>the number of divided wells is  $0.9956^{160} = 0.4938$ . For example, if the acceptable probability of false-negative results is 1%, the following calculation can be made:  $n = \log(0.01)/\log(0.4938) = 6.526$ . In other words, no colonies should be detected when an assay similar to the one performed for the positive control cells is run 7 times in order to demonstrate that malignant transformed cells equivalent to HeLa cells are not intermingled with a cellular sample at a ratio of  $1/10^7$ .

## Reference Information 8: Characterization of cell proliferation as a method to detect transformed cells intermingled with product cells

Source 1: Kono K, Takada N *et al.* Characterization of the cell growth analysis for detection of immortal cellular impurities in human mesenchymal stem cells. *Biologicals*. 2015;43:146-9. (See also: Kono K, Takada N *et al.* Corrigendum to "Characterization of the cell growth analysis for detection of immortal cellular impurities in human mesenchymal stem cells" [Biologicals 43 (2) (March 2015) 146-149]. *Biologicals*. 2017;45:106.)

Source 2: Hasebe-Takada N, Kono K *et al.* Application of cell growth analysis to the quality assessment of human cell-processed therapeutic products as a testing method for immortalized cellular impurities. *Regen Ther*. 2016;5:49-54.

## Methodology

### 1. Cells

Human bone marrow-derived mesenchymal stem cells (hMSCs) are cultured in the mesenchymal stem cell growth medium (MSCGM) up to the 5th passage. Human adipose-derived stem cells (ADSCs) are cultured in the ADSC-BulletKit up to the 5th passage. HeLa cells are cultured in the Eagle's minimum essential medium spiked with 10% fetal bovine serum (FBS), 0.1 mM non-essential amino acid solution, 50 U/mL penicillin, and 50 mg/mL streptomycin. hTERT-immortalized adipose-derived mesenchymal stem cells (ASC52telo) are cultured in the ADSC-BulletKit.

### 2. Characterization of Cell Proliferation

To  $1 \times 10^6$  of hMSCs at passage 5, add HeLa cells at a concentration of  $10^3$  (0.1%),  $10^2$  (0.01%), 10 (0.001%), and 1 (0.0001%), respectively, and seed in a T175 flask. Or, to  $1 \times 10^6$  of ADSCs at passage 5, add HeLa cells at a concentration of  $10^3$  (0.1%),  $10^2$  (0.01%), and 10 (0.001%), respectively, and seed in a T175 flask. Culture the cells in 40 mL of a medium, which is Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 50 U/mL penicillin, and 50 mg/mL streptomycin, while replacing the medium with fresh medium every 2 or 3 days. Wash cells reaching approximately 90% confluence with phosphate-buffered saline (PBS), and detach them from the flask with a 0.05% trypsin-EDTA solution. Centrifuge the recovered cells for 5 minutes at  $450 \times g$  to remove culture supernatant, and suspend the cells in a fresh medium. Stain aliquots of the suspended cells with a trypan blue solution and count the number of cells with an automated cell counter. Seed  $1 \times 10^6$  cells in a T175 flask and culture until next passage. Repeat the above procedure up to the 10th passage (hMSCs spiked with HeLa cells) or the 20th passage (ADSCs spiked with ASC52telo cells). Calculate the cell proliferation rate using the following formula:

$$R_n = [\log_2(N_{n+1} / N_n)] / (D_{n+1} - D_n)$$

$N_k$ ; the number of accumulated cells at the passage  $k$ ,  $D_k$ ; the date at the passage  $k$

Determine contamination of the cultured cells with immortalized cells based on the presence or absence of a significant difference in cell proliferation rate compared with the 5th passage or with the negative control.