FULL-LENGTH ARTICLE

Regulatory Policies

Multisite studies for validation and improvement of a highly efficient culture assay for detection of undifferentiated human pluripotent stem cells intermingled in cell therapy products

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ABSTRACT

Background aims: The Multisite Evaluation Study on Analytical Methods for Non-Clinical Safety Assessment of Human-Derived Regenerative Medical Products (MEASURE) is a Japanese experimental public–private partnership initiative, which aims to standardize methodology for tumorigenicity evaluation of human pluripotent stem cell (hPSC)-derived cell therapy products (CTPs). Undifferentiated hPSCs possess tumorigenic potential, and thus residual undifferentiated hPSCs are one of the major hazards for the risk of tumor formation from hPSC-derived CTPs. Among currently available assays, a highly efficient culture (HEC) assay is reported to be one of the most sensitive for the detection of residual undifferentiated hPSCs.

Methods: MEASURE first validated the detection sensitivity of HEC assay and then investigated the feasibility of magnetic-activated cell sorting (MACS) to improve sensitivity.

Results: The multisite experiments confirmed that the lower limit of detection under various conditions to which the human induced pluripotent stem cell lines and culture medium/substrate were subjected was 0.001%. In addition, MACS concentrated cells expressing undifferentiated cell markers and consequently achieved a detection sensitivity of 0.00002%.

Conclusions: These results indicate that HEC assay is highly sensitive and robust and that the application of MACS on this assay is a promising tool for further mitigation of the potential tumorigenicity risk of hPSC-derived CTPs.

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Introduction

Human pluripotent stem cells (hPSCs), such as human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells, have the infinite capacity to self-renew and differentiate into any cell

type in the body [1–4]. Therefore, hPSC-derived cell therapy products (CTPs) are expected to offer promising treatments for a wide variety of diseases for which no adequate therapy is currently available. Despite this extraordinary potential, hPSCs are intrinsically tumorigenic and form teratomas in immunodeficient animals when they are introduced in their pluripotent state [5–7]. Hentze *et al.* [8] previously reported that a few hundred undifferentiated human embryonic stem cells were sufficient to produce teratomas in severe combined immunodeficient mice. Recently, studies using severely immunodeficient mice, such as NOD/SCID/

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γ^{Cnull} mice [9], revealed that 10–100 undifferentiated hiPSCs caused teratomas when hiPSCs were subcutaneously injected with Matrigel [10]. Though these abnormalities were seen under conditions where hiPSCs were embedded in a basement membrane matrix to increase their tumor-forming potential, a trace amount of residual undifferentiated hPSCs in hPSC-derived CTPs may lead to tumor development following transplantation. During the manufacturing process, most undifferentiated hPSCs are likely eliminated, as most differentiation protocols are not optimal for survival of undifferentiated hPSCs [11]. In addition, a variety of approaches have been reported for selective elimination of undifferentiated hPSCs, including the use of cytotoxic antibodies, to decrease the risk of teratoma formation in hPSC-derived CTPs [12–14]. However, rigorous assessment for contamination with undifferentiated hPSCs is mandatory to mitigate the potential tumorigenicity risks of hPSC-derived CTPs [5,9,10,15–19].

As just described, an *in vivo* teratoma formation assay using severely immunodeficient mice is the conventional method used to directly assess residual undifferentiated hPSCs, but this assay is costly and time-consuming [6]. Several *in vitro* methods to detect pluripotency marker proteins or genes using flow cytometry [20] or quantitative real-time polymerase chain reaction (qRT-PCR) [20] and droplet digital PCR (ddPCR) [21] have been reported. In addition, Tatenio *et al.* [22] established a sandwich assay system called the GlycoStem test, which can quantitatively detect residual hPSCs using pluripotent stem cell-specific lectin probe rBC2LCN (the recombinant N-terminal domain of BC2L-C). These *in vitro* assays are simple and robust; however, the limit of detection (LOD) of assays is relatively high and reported to be 0.1% and 0.05% for flow cytometry [20] and the GlycoStem test [22], respectively. In addition, with regard to flow cytometry, gating techniques strongly influence the results. Although PCR-based assays are more sensitive (the LOD is 0.002% for qRT-PCR assay [20] and 0.001% for ddPCR [21]), it is known that some types of hiPSC-derived cells also retain the expression of pluripotency marker genes during the differentiation process [23], and this may imply that the marker gene for PCR-based assays needs to be carefully validated prior to the assay for each CTP. A highly efficient culture (HEC) assay, first reported by Tano *et al.* [23], is able to detect colonies formed from hPSCs using a highly efficient culture system, which favors the growth of hPSCs. This assay requires about a 1-week culture period; however, the assay can directly detect hPSCs by identifying the colony derived from a single hPSC and is highly sensitive: the LOD is reported to be 0.001% [23].

The Multisite Evaluation Study on Analytical Methods for Non-Clinical Safety Assessment of Human-Derived Regenerative Medical Products (MEASURE) is a Japanese experimental public–private partnership initiative launched by the National Institute of Health Sciences and the Committee for Non-Clinical Safety Evaluation of Pluripotent Stem Cell-Derived Product of the Forum for Innovative Regenerative Medicine with the goal of standardizing the tumorigenicity-associated testing methods for hPSC-derived CTPs [24].

Throughout its activities, MEASURE has selected HEC assay as one of the most efficient *in vitro* assays for detection of residual undifferentiated hPSCs and carried out a comprehensive evaluation of its feasibility [24]. In the present study, as a subgroup of MEASURE, the authors first validated the reproducibility of the reported LOD of HEC assay using several hiPSC lines under various culture medium/substrate conditions at a number of facilities and according to a standardized protocol and then investigated the feasibility of several markers for hiPSC colony identification. In addition, the authors developed a methodology to concentrate undifferentiated hPSCs in CTPs by utilizing magnetic-activated cell sorting (MACS) and validated this methodology by applying it to HEC assay for further improvement of assay sensitivity.

Methods

Cells

The hiPSC lines 201B7, 1231A3 and Cellartis human iPS cell line 18 (ChiPSC18) were used in this study (see supplementary Table 1). The 201B7 cells were obtained from the RIKEN BioResource Research Center or the Center for iPS Cell Research and Application, Kyoto University. The 1231A3 cells were provided by the Center for iPS Cell Research and Application. The ChiPSC18 cells were purchased from Takara Bio Inc. The 201B7 and 1231A3 cells were maintained in defined culture medium StemFit AK03N (Ajinomoto Co, Inc) in accordance with the reported procedure [25], with minor modifications.

Every 6–7 days, at 80% confluency, cells were passaged by treatment with a detachment solution consisting of $\times 0.5$ TrypLE Select or TrypLE Express ($\times 1$ TrypLE Select or $\times 1$ TrypLE Express [Thermo Fisher Scientific] diluted one-half with 0.5 mM ethylenediaminetetraacetic acid/phosphate-buffered saline [PBS] [Nacalai Tesque Inc] to a final concentration of 0.75 mM ethylenediaminetetraacetic acid) for 5–10 min at 37°C. Cells were seeded at 2×10^4 cells/cm² onto culture dishes in StemFit AK03N medium supplemented with 10 μ M ROCK inhibitor Y-27632 (Fujifilm Wako Pure Chemical Corp) and iMatrix-511 solution (Nippi Inc) prepared for 0.25 μ g/cm². Following recovery for 24 h, cells were fed with StemFit AK03N medium alone for the remainder of the culture. The volume of culture medium applied to culture dishes was adjusted to 200 μ L/cm². ChiPSC18 cells were maintained with the Cellartis DEF-CS culture system (Takara Bio Inc) according to the manufacturer's instructions. When passaged every 3–7 days, cells were detached by treatment with $\times 1$ TrypLE Select or $\times 1$ TrypLE Express for 4–5 min at 37°C and reseeded at 1×10^4 cells/cm² on culture dishes coated with DEF-CS COAT-1 in the DEF-CS medium. The cultured hiPSCs 201B7 (passages 24–39), 1231A3 (passages 14–18) and ChiPSC18 (passages 20–22) were frozen in Stem-Cellbanker (Zenoq Resource Co, Ltd) and cryopreserved in liquid nitrogen. The frozen hiPSCs were thawed and used for experiments after two to eight passages.

Poietics™ Normal Human Bone Marrow Derived Mesenchymal Stem Cells (hMSCs, human mesenchymal stromal cells) were purchased from Lonza (#PT-2501) and cultured in MSCGM medium (Lonza). The hMSCs from passages four to six were used in this study. Human peripheral blood mononuclear cells (hPBMCs) were purchased from Lonza (#CC-2702). To generate human T cells, hPBMCs were suspended in X-VIVO 10 medium (Lonza) supplemented with 100 ng/mL recombinant human IL-2 (PeproTech), 10% fetal bovine serum (Thermo Fisher Scientific) and 20 U/mL DNase I (Qiagen N.V.) and then plated on the dishes pre-coated with 10 μ g/mL mouse anti-human CD3 antibody (#555336; BD Biosciences). Generated human T cells from passage two were used in this study. All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

HEC assay

The hiPSCs 201B7, 1231A3 and ChiPSC18 were dissociated into single cells with TrypLE Express, and after confirming the viability ($> \sim 80\%$) by Trypan Blue exclusion with the Countess automated cell counter (Thermo Fisher Scientific) using a sensitivity setting of 5, minimum size of 8, maximum size of 30 and circularity of 75, these cells were suspended in Essential 8 Flex medium (Thermo Fisher Scientific) or StemFit AK03N, each containing 10 μ M Y-27632 (E8F+Y medium or AK03N+Y medium). The cell suspension of hMSCs was prepared in the same manner and mixed with each hiPSC cell suspension to prepare suspensions containing 10 (0.001%) or 30 (0.003%) hiPSCs in 1×10^6 hMSCs. More specifically, 3×10^6 cells/2.4 mL hMSC cell suspension was mixed with 0.6 mL of 50 cells/mL (0.001%) or 150 cells/mL (0.003%) hiPSC cell suspension, which was carefully

prepared by serial dilution from 5×10^5 cells/mL of the hiPSC cell suspension at a ratio of 10 or 33.3. Each 1 mL of cell suspension using E8F+Y medium or AK03N+Y medium was respectively seeded on 10-cm culture dishes coated with laminin-521 (BioLamina AB) [23] or 10-cm culture dishes containing iMatrix-511 solution ($0.25 \mu\text{g}/\text{cm}^2$) [25]. The day after the seeding, media were replaced with each respective medium without Y-27632, and after 5 or 6 days of incubation at 37°C in 5% CO_2 , the dishes were fixed with 4% paraformaldehyde phosphate buffer solution and stained using a Vector Blue Alkaline Phosphatase (ALP) substrate kit (Vector Laboratories, Inc) according to the manufacturer's instructions. The ALP-positive hiPSC colonies were counted manually by two different operators under a microscope. These assays were conducted at four facilities (facilities A, B, C and D), and each assay was carried out in duplicate and repeated three times at each facility.

The true positive rate (TPR) for detection of hiPSC colonies with the HEC assay was calculated based on the assumption that the number of hiPSC colonies formed on culture dishes follows a Poisson distribution [26]. The formula for the Poisson distribution with parameter $\lambda > 0$ is given as follows:

$$P(X = k) = e^{-\lambda} \lambda^k / k! \text{ for } k = 0, 1, 2, \dots$$

When this formula is applied to the authors' models to calculate the probability of detecting hiPSC colonies, the parameters k and λ are respectively thought to correspond to the specified number of hiPSC colonies in question and the expected number of hiPSC colonies per experiment. The probability of no hiPSC colony formation ($k = 0$)—i.e., the false-negative rate (FNR)—in a single experiment was expressed using the mean number of actual hiPSC colonies obtained by repeated experiments ($\lambda = n$):

$$P_{\text{sgl}}(X = 0) = e^{-n} \times n^0 / 0! = e^{-n}$$

The FNR of a set of three experiments was calculated as the probability of no hiPSC colony formation in a single experiment (e^{-n}) raised to the third power:

$$P_{\text{trpl}}(X = 0) = e^{-n \times 3} = e^{-3n}$$

The probability of hiPSC colony formation ($k > 0$)—i.e., TPR—in a single experiment and a set of three experiments led to the calculation one minus FNR:

$$P_{\text{sgl}}(X > 0) = 1 - P_{\text{sgl}}(X = 0)$$

$$P_{\text{trpl}}(X > 0) = 1 - P_{\text{trpl}}(X = 0)$$

In this study, use of the term *TPR* was identical to “clinical/diagnostic sensitivity,” as defined in ISO/TS 17822-1:2014 [27], whereas the term *sensitivity* was used in a broad sense.

Lectin staining and immunostaining

The ChiPSC18-derived colonies, which were cultured in AK03N+Y medium and iMatrix-511 (AK03N/iMatrix), as noted previously, were fixed with 4% paraformaldehyde, and lectin staining and immunostaining were performed as follows. For lectin staining, endogenous peroxidase activity was blocked by pre-incubation with 3% hydrogen peroxide in PBS for 5 min at room temperature, and after washing with PBS, dishes were incubated with 1:100 peroxidase-labeled rBC2LCN (Fujifilm Wako Pure Chemical Corp) for 1 h at room temperature. After washing with PBS, staining was developed with 3,3'-diaminobenzidine substrate. For immunostaining, endogenous peroxidase activity was blocked by pre-incubation with 3% hydrogen peroxide in PBS for 5 min at room temperature, and then cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. After blocking with Blocking One (Nacalai Tesque, Inc) for 1 h at room temperature, dishes were incubated with 1:250 biotin-

labeled anti-TRA-1-60 antibody (#13-8863-82; Thermo Fisher Scientific) or 1:500 biotin-labeled anti-SSEA-4 antibody (#330404; BioLegend) at 4°C overnight. After washing with PBS, 1:500 streptavidin horseradish peroxidase (#405210; BioLegend) was added to dishes for 1 h at room temperature, and then staining was developed with ImmPACT 3,3'-diaminobenzidine (Vector Laboratories, Inc).

Furthermore, hiPSC colonies formed under the aforementioned culture condition of AK03N/iMatrix (ChiPSC18, 0.003%) were restained with peroxidase-labeled rBC2LCN following ALP staining. The rBC2LCN-positive colonies were also counted at each facility (facilities A, B, C and D).

Magnetic-activated cell sorting

The sorting efficiency of spiked hiPSCs by the MACS system using microbeads conjugated with anti-pluripotent stem cell markers was investigated using the ddPCR method. The ChiPSC18 and human T cells expanded from hPBMCs were dissociated, and cell suspensions containing 25 (0.0025%) hiPSCs in 1×10^6 human T cells were prepared using AK03N+Y medium. The cell suspension was spun down at $300 \times g$ for 10 min at room temperature. After centrifugation, supernatant was discarded, and the cell pellets were reconstituted in $40 \mu\text{L}$ AK03N+Y medium and incubated with $10 \mu\text{L}$ anti-human TRA-1-60 microbeads (Miltenyi Biotec) at 4°C for 5 min. The suspensions were then reconstituted in 3 mL AK03N+Y medium and applied to an LS column attached to a MidiMACS separator (Miltenyi Biotec). After the column was washed with AK03N+Y medium, TRA-1-60-positive cells retained inside the column were flushed out very gently with 3 mL AK03N+Y medium. After centrifugation at $300 \times g$ for 15 min, cells from both eluted and flow-through fractions were dissolved in $350 \mu\text{L}$ buffer RLT and stored at -70°C until analysis.

Total RNA was isolated using an RNeasy micro or mini kit with DNase I treatment (Qiagen N.V.) according to the manufacturer's instructions. RNA quantity was determined using a NanoDrop One (Thermo Fisher Scientific). The presence of hiPSCs in RNA samples of eluted fractions (total amount of one fraction) and flow-through fractions (50 ng) was analyzed using the one-step ddPCR method with a probe and primers targeting *LIN28A* transcripts according to the previously reported procedure [21]. Twenty microliters of reaction mixtures containing total RNA, a 250-nM probe and 900-nM forward and reverse primers were prepared with the One-Step RT-ddPCR advanced kit for probes (Bio-Rad Laboratories, Inc). The sequences of the probe and primers (Sigma-Aldrich; Merck KGaA) were as follows: probe, 5'-FAM-CGCATGGGGTTCGGCTTCTGTCC-BHQ-1-3'; forward primer, 5'-CACGGTGCGGGCATCTG-3'; reverse primer, 5'-CCTTCCATGTGCAGCTTACTC-3'.

Droplets were generated in DG8 cartridges (Bio-Rad Laboratories, Inc) using the QX100 droplet generator (Bio-Rad Laboratories, Inc) according to the manufacturer's instructions. Water-in-oil emulsions were transferred to a 96-well plate, and RT-PCR was performed using a T100 thermal cycler (Bio-Rad Laboratories, Inc). Thermal cycling conditions were as follows: 30 min reverse transcription at 60°C , followed by 5 min enzyme activation at 95°C and 40 cycles of a thermal profile comprising 30-second denaturation at 94°C and 60-second annealing/extension at 58°C . After PCR amplification, products were denatured at 98°C for 10 min and cooled at 4°C . The fluorescence intensities of each droplet from the samples were measured using the QX100 droplet reader (Bio-Rad Laboratories, Inc). Positive droplets containing amplification products were distinguished from negative droplets and counted by applying a fluorescence amplitude threshold in QuantaSoft software (Bio-Rad Laboratories, Inc). The threshold was manually determined at the highest point of the sample droplet cluster of water. Total RNA isolated from hiPSCs was used as the positive control in the ddPCR assay.

After confirmation of highly efficient sorting of hiPSCs by this MACS system with ddPCR, 2 mL of cell suspensions containing 10 (0.00002%) or 25 (0.00005%) hiPSCs in 5×10^7 human T cells was

incubated with 500 μ L anti-human TRA-1-60 microbeads at 4°C for 5 min, reconstituted in 6 mL AK03N+Y medium and applied to an LS column. After the column was washed, TRA-1-60-positive cells retained inside the column were flushed out very gently with 3 mL AK03N+Y medium, and the eluted suspensions were seeded into six-well culture dishes containing iMatrix-511 solution. Two or 3 days after the seeding, the medium was replaced with StemFit AK03N without Y-27632, and after 7–10 days of incubation, ALP-positive hiPSC colonies were counted manually under a microscope.

These assays were conducted at three facilities (facilities A, B and C), and each assay was carried out in duplicate and repeated three times at each facility. The exception to this is the ddPCR analysis, for which all lysate samples from each facility were delivered to facility E and were analyzed at this facility.

Statistical analysis

For the experiment regarding reproducibility of the reported LOD of HEC assay, the variation in the number of hiPSC colonies among hiPSC lines and different culture conditions was statistically analyzed for each number of spiked hiPSCs and each facility. Tukey's test was performed to compare the mean among hiPSC lines, and Student's *t*-test was performed to compare the mean between the culture conditions. For the experiment regarding improvement of assay sensitivity by MACS, ddPCR data were analyzed by two-way analysis of variance, followed by Tukey's post hoc test, and the variation in the number of hiPSC colonies among facilities was analyzed for each number of spiked hiPSCs by Tukey's test. Each test was conducted using two-tailed significance levels of 0.05 and 0.01. Analyses were performed using SAS 9.3 (SAS Institute Inc). Prism 8 (GraphPad Software, LLC) was used to display the results as a box plot.

Results

Reproducibility of the reported LOD of HEC assay

The mean number of ALP-positive hiPSC colonies from three duplicate experiments of each hiPSC line or culture condition is shown in Table 1 and Figure 1. The hiPSC colonies were confirmed in one or both duplicate dishes at each experiment in both the 10 (0.001%) and 30 (0.003%) hiPSC-spiked conditions of all three hiPSC lines (201B7, 1231A3 and ChiPSC18) under each culture condition (E8F/LN521 or AK03N/iMatrix) at all four facilities. However, no colonies were observed in duplicate dishes in two out of three experiments under the 10 hiPSC-spiked condition of 1231A3 using AK03N/iMatrix at facility D. The mean number of hiPSC colonies under the 30 hiPSC-spiked condition of 1231A3 using AK03N/iMatrix at facility D showed a statistically significant lower value compared with that seen under the same condition of 201B7 or ChiPSC18. However, no significant differences among hiPSC lines were seen in the other facilities, apart from facility D, or when E8F/LN521 was used. Therefore, hiPSC colony formation did not seem to be different among hiPSC lines under both of the culture conditions used in this study.

In addition, the mean number of hiPSC colonies cultured in E8F/LN521 was statistically significantly higher or lower than the mean number of hiPSC colonies cultured in AK03N/iMatrix in a few cases involving hiPSC lines and facilities. However, these variations did not show consistent trends, indicating that hiPSC colony formation was also equivalent between these two culture conditions. Thus, there were no clear differences in the number of hiPSC colonies between the two culture conditions. To confirm the TPR needed to detect spiked hiPSCs under the experimental conditions, the authors used the Poisson distribution formula and calculated the probability of detecting hiPSC colonies with one or more colonies present. The TPR of a single experiment varied in facilities on spiking with 10 (0.001%) hiPSCs regardless of hiPSC line or culture condition, but the mean

Table 1

Number of ALP-positive hiPSC colonies after HEC assay using various hiPSC lines under a range of culture conditions.

Culture condition	E8F/LN521		AK03N/iMatrix	
	Number (%) of spiked hiPSCs			
	10 (0.001)	30 (0.003)	10 (0.001)	30 (0.003)
ChiPSC18				
Facility A	6.0 ± 2.0	16.9 ± 4.2	5.3 ± 1.3	13.8 ± 1.3
Facility B	0.8 ± 0.5	4.9 ± 0.8 ^a	2.0 ± 0.9	7.5 ± 1.1
Facility C	1.5 ± 0.9	14.5 ± 2.9	1.8 ± 0.5	15.1 ± 1.9
Facility D	4.0 ± 1.8	12.0 ± 1.7	3.0 ± 1.2	9.6 ± 1.2
201B7				
Facility A	3.3 ± 0.8	7.3 ± 2.4	4.2 ± 2.4	6.8 ± 1.1
Facility B	1.3 ± 0.6	4.8 ± 4.0	2.5 ± 1.0	6.6 ± 1.2
Facility C	1.2 ± 0.6	12.4 ± 0.5 ^a	1.6 ± 0.7	14.1 ± 0.4
Facility D	3.0 ± 0.5	9.5 ± 0.8	3.0 ± 1.8	8.1 ± 3.2
1231A3				
Facility A	5.4 ± 5.3	11.5 ± 6.1	6.7 ± 5.6	11.3 ± 7.7
Facility B	1.6 ± 0.7	3.4 ± 0.8 ^b	2.5 ± 1.8	7.9 ± 1.3
Facility C	1.3 ± 0.5	15.2 ± 3.3	1.6 ± 1.0	14.5 ± 1.6
Facility D	1.8 ± 0.9	9.6 ± 1.0 ^b	0.3 ± 0.4	1.5 ± 0.9 ^{c,d}

Either 10 or 30 hiPSCs (201B7, 1231A3 or ChiPSC18) were spiked into 1×10^6 hMSCs and cultured in E8F/LN521 or AK03N/iMatrix using 10-cm culture dishes. After 5 or 6 days of incubation, ALP-positive hiPSC colonies were counted manually under a microscope. These assays were conducted at four facilities (facilities A, B, C and D), and values (mean $\pm$ SD) were obtained from three duplicate experiments. Variations in number of hiPSC colonies among hiPSC lines under different culture conditions were statistically analyzed for each number of spiked hiPSCs and the facility. Tukey's test was performed to compare the mean among hiPSC lines, and Student's *t*-test was performed to compare the mean between the culture conditions.

SD, standard deviation.

^a *P* < 0.05 versus AK03N/iMatrix.

^b *P* < 0.01 versus AK03N/iMatrix.

^c *P* < 0.01 versus ChiPSC18.

^d *P* < 0.05 versus 201B7.

values at all the facilities were approximately 80% or higher; spiking with 30 (0.003%) hiPSCs gave a TPR of approximately 100% at almost all facilities (Figure 2A). Three repeated experiments increased the TPR for detection of hiPSC colonies, and a TPR of approximately 100% was calculated at most facilities on spiking with 10 cells (Figure 2B). These findings indicate that a negative result obtained from a product with an unknown contamination rate means “the hiPSC content is less than 0.001%” at a probability of 80% or higher, even with a single experiment.

The morphology of the hiPSC colonies was slightly different between E8F/LN521 and AK03N/iMatrix. Namely, under the condition

Figure 1. Number of ALP-positive hiPSC colonies after HEC assay. Either 10 (0.001%) or 30 (0.003%) hiPSCs were spiked into 1×10^6 hMSCs. The number of detected colonies under various conditions is indicated by box plots with Tukey whiskers. Data represent the results of three duplicate experiments from four different facilities under certain conditions. Median values are marked by horizontal lines. Boxes indicate 25–75% data ranges, and the range outside 1.5 times the IQR is shown as whiskers. Outliers are plotted as open circles. IQR, interquartile range.

Figure 2. TPR for detecting hiPSC colonies using HEC assay. Either 10 (0.001%) or 30 (0.003%) hiPSCs were spiked into 1×10^6 hMSCs. Bar graphs show the TPR for detecting hiPSC colonies at four facilities with a single (A) experiment and three repeated (B) experiments. Error bars are mean $\pm$ SD. SD, standard deviation.

of AK03N/iMatrix, some colonies showed a tendency toward differentiation in the peripheral area of the colony, with reduced intensity of ALP staining (Figure 3). This tendency was consistently observed among all hiPSC lines at all facilities and was possibly related to the more prominent proliferation of hMSCs, which was noted in the dishes under the condition of AK03N/iMatrix.

Lectin staining and immunostaining

The hiPSC colonies were clearly stained and identified by lectin staining with rBC2LCN and immunostaining with anti-TRA-1-60 or anti-SSEA-4 antibody (Figure 4). The intensities of these stainings were similar to those seen with ALP staining, whereas there was some difference in the staining intensity of the peripheral area of the colony among these staining methodologies. The colonies showed

partial differentiation in the peripheral area of the colony cultured in AK03N/iMatrix, and these areas showed reduced intensity of ALP staining. The anti-TRA-1-60 antibody stained only a few cells in the peripheral area of the colony; however, rBC2LCN and anti-SSEA-4 antibody stained these cells moderately. Moreover, ALP staining and immunostaining with anti-SSEA-4 antibody showed that background staining originated in some hMSCs. However, the background staining of hMSCs was negligible when staining with rBC2LCN and anti-TRA-1-60 antibody. The mean number of rBC2LCN-positive hiPSC colonies, counted using the same dishes with which ALP-positive hiPSC colonies were counted, was 14.0 ± 1.1 , 8.8 ± 2.0 , 15.1 ± 1.9 and 13.7 ± 2.4 at facilities A, B, C and D, respectively (see supplementary Table 2), and was equivalent to or slightly higher than the mean number of ALP-positive hiPSC colonies (13.8 ± 1.3 , 7.5 ± 1.1 , 15.1 ± 1.9 and 9.6 ± 1.2 at facilities A, B, C and D, respectively, in Table 1; ChiPSC18, 30 cells, culture condition of AK03N/iMatrix).

Improvement of assay sensitivity by MACS

To improve the ability to detect residual undifferentiated hPSCs in CTPs, the authors attempted to combine HEC assay with the MACS system, concentrating hiPSCs with anti-human TRA-1-60 microbeads. First, at three facilities, the authors examined whether anti-human TRA-1-60 microbeads trapped 25 hiPSCs (0.0025%) suspended in 1×10^6 human T cells using the one-step ddPCR method targeting *LIN28A* transcripts, which are exclusively expressed in hPSCs [21]. The numbers of positive droplets (i.e., copy numbers of *LIN28A* messenger RNA) in the eluted fractions were significantly high compared with the flow-through fractions by two-way analysis of variance ($P = 0.001$) even though total RNA used in the ddPCR assay of the eluted fractions was undetectable with a spectrophotometer and likely much less than that of the flow-through fractions (Figure 5). A relatively large standard deviation value was noted in the number of positive droplets in the eluted fraction at facility C, possibly due to the variation in number of spiked hiPSCs, viability of hiPSCs during manipulation and/or efficiency in trapping hiPSCs with magnetic beads. However, no significant difference in positive droplet number among facilities was observed in either the flow-through or eluted fraction (Table 2). These results suggest that anti-human TRA-1-60 microbeads selectively bound to undifferentiated hiPSCs mixed with human T cells and successfully concentrated the hiPSCs.

Next, the authors investigated the efficiency of hiPSC colony formation after sorting with anti-TRA-1-60 microbeads. Either 10 (0.00002%) or 25 hiPSCs (0.00005%) spiked into 5×10^7 human T cells were sorted by anti-human TRA-1-60 microbeads, and hiPSC colonies formed after incubation were counted. As a result, hiPSC colonies were confirmed in at least one of the duplicate dishes in each

Figure 3. ALP-positive hiPSC colonies after HEC assay. Morphologies of ALP-positive-forming colonies derived from 201B7 spiked into hMSCs under culture conditions E8F/LN521 (A) and AK03N/iMatrix (B) are shown. Colonies cultured in AK03N/iMatrix showed a tendency toward differentiation in the peripheral area, with reduced ALP staining intensity. Scale bars are 200 μ m.

Figure 4. Lectin staining and immunostaining of hiPSC colonies. ChiPSC18-derived colonies were stained with peroxidase-labeled rBC2LCN (A) for lectin staining and with biotin-labeled anti-TRA-1-60 (B) or anti-SSEA-4 (C) antibody and streptavidin horseradish peroxidase for immunostaining. Colonies were clearly stained by all staining methodologies, and the staining intensity was sufficient for counting. Scale bars are 300 μ m.

experiment in both the 10 (0.00002%) and 25 (0.00005%) hiPSC-spiked conditions at all three facilities. The mean number of hiPSC colonies did not show a statistically significant difference among facilities for either the 10 or 25 hiPSC-spiked condition (Table 3). The mean TPR values for a single experiment and for three repeated experiments using HEC assay at three facilities were 90% or more on spiking with 10 and 25 cells (Table 4).

Discussion

HEC assay is a culture assay that can directly detect a trace amount of hiPSCs by identifying the colony derived from a single hiPSC. This assay is highly sensitive, and Tano *et al.* [23] reported that the LOD was 0.001% when a 10-cm dish was used as a culture vessel. Indeed, this assay was employed in the tumorigenicity assessment of an hPSC-derived cell therapy product, human parthenogenetic stem

cell-derived neural stem cells (ISC-hpNSCs) prior to conducting a phase 1 clinical trial under the requirements of the US Food and Drug Administration and the Australian Therapeutic Goods Administration (clinicaltrials.gov identifier NCT02452723) [28]. Briefly, ISC-hpNSCs were grown either as a pure population (100% ISC-hpNSCs) or spiked with 0.1%, 0.01% or 0.001% hPSCs. After 1 week of culture, the presence of hPSC colonies or OCT-4 expression was confirmed under the spiked 0.1%, 0.01% and 0.001% hPSC culture conditions. By contrast, neither hPSC colonies nor OCT-4 expression was detected in the pure, unspiked ISC-hpNSC culture, and thus it was concluded that the residual hPSC content in ISC-hpNSCs is below 0.001% [28].

As a first step, the authors of the current study investigated the reproducibility of the reported LOD (0.001%) of this assay using various hiPSC lines under a range of culture conditions. As in the study by Tano *et al.* [23], hiPSC line 201B7 was spiked into hMSCs and

Fig. 5. Copy numbers of *LIN28A* mRNA investigated by ddPCR. Three experiments utilizing ddPCR are divided by vertical dotted yellow lines. The purple line represents the fluorescence amplitude threshold applied to the analysis for designating positive or negative droplets. The blue dots are positive for *LIN28A* mRNA, and the gray dots are negative for *LIN28A* mRNA. mRNA, messenger RNA.

Table 2

LIN28A mRNA copy numbers determined by ddPCR.

Fraction	Facility A	Facility B	Facility C
Flow-through	10.3 $\pm$ 3.2	5.3 $\pm$ 5.0	5.3 $\pm$ 2.5
Elution	74.0 $\pm$ 3.5	84.7 $\pm$ 20.5	133.3 $\pm$ 104.6

Values (mean $\pm$ SD) were obtained from three duplicate experiments.

mRNA, messenger RNA; SD, standard deviation.

Table 3

Detection sensitivity of HEC assay after MACS with microbeads conjugated with anti-human TRA-1-60.

	Number (%) of spiked hiPSCs (ChiPSC18)	
	10 (0.00002)	25 (0.00005)
Facility A	2.3 $\pm$ 1.0	8.8 $\pm$ 1.5
Facility B	1.8 $\pm$ 1.2	6.2 $\pm$ 2.8
Facility C	3.2 $\pm$ 1.5	7.0 $\pm$ 4.0

Values (mean $\pm$ SD) were obtained from three duplicate experiments.

SD, standard deviation.

Table 4

TPR for detecting hiPSC colonies using HEC assay after MACS.

	Number (%) of spiked hiPSCs (ChiPSC18)	
	10 (0.00002)	25 (0.00005)
$\times$ 1 experiment	90 $\pm$ 6	100 $\pm$ 0
$\times$ 3 experiments	100 $\pm$ 0	100 $\pm$ 0

Values (%) are mean $\pm$ SD from three facilities with a single ($\times$ 1) experiment and three repeated ($\times$ 3) experiments.

SD, standard deviation.

cultured in Essential 8 medium on laminin-521-coated 10-cm culture dishes. In the present study, two additional hiPSC lines, 1231A3 and ChiPSC18, were also spiked into hMSCs (10 or 30 hiPSCs in 1×10^6 hMSCs, 0.001% or 0.003%, respectively) and cultured in Essential 8 medium on laminin-521-coated 10-cm culture dishes or in StemFit AK03N on 10-cm culture dishes containing iMatrix-511 solution. These studies were performed at four facilities, and the hiPSC colonies were observed in dishes under both the 10 and 30 hiPSC-spiked conditions at all facilities regardless of hiPSC line or culture condition. Although the number of hiPSC colonies showed slight variation among hiPSC lines and culture conditions, the statistically significant difference seen sporadically in some hiPSC lines and culture conditions did not reveal a consistent trend that would indicate specific effects of cell line or culture condition on hiPSC colony formation. These results clearly demonstrate that the reported LOD of HEC assay (0.001% under the condition where a 10-cm culture dish was used) is reproducible regardless of hiPSC line or culture condition, suggesting that HEC assay is a robust and sensitive method for detecting residual undifferentiated hPSCs in CTPs.

It should be noted that no colony formation was seen with a relatively high incidence in the specific situation where hiPSC line 1231A3 was cultured in AK03N/iMatrix at facility D. The reason for the low colony formation probability is not clear, but a similar event could occur during the actual evaluation of hPSC-derived CTPs. In a spiking experiment where the hPSC line is used as raw material for the product, it is desirable to define the LOD of the assay for each CTP at each test facility. The TPR for detecting 0.001% hiPSC contamination was approximately 80% or greater, even with a single experiment, in the MEASURE multisite study. However, if a low colony formation rate is supposed during the assay validation, increasing the number of dishes per experiment and/or repeating experiments to confirm actual LOD on a case-by-case basis should be considered. The hiPSCs in the peripheral area of the colonies formed on the dishes with AK03N/iMatrix seemed to show slight differentiation, for which the intensity of ALP staining was reduced. However, this did not affect hiPSC colony identification or the counted number of colonies in the present experiments. Excessive cell density due to prominent proliferation of hMSCs, a phenomenon that was also consistently observed when colonies were cultured in AK03N/iMatrix, may relate to the differentiation profile of hiPSCs. Therefore, in a case where hiPSC colonies show more pronounced differentiation, which may affect colony identification, appropriate modification to maintain suitable cell density (i.e., decreased number of CTPs per dish) should be considered.

In the case of manual colony counting, bright field observation is considered more convenient, and in this respect, ALP is one of the more useful markers for HEC assay. However, when CTPs themselves express ALP—for example, bone or cartilage CTPs—this marker should not be used to identify hiPSC colonies. Staining with pluripotent stem cell-specific lectin probe rBC2LCN and with antibodies to pluripotent stem cell markers (TRA-1-60 and SSEA-4) can clearly identify hiPSC colonies; therefore, these staining methodologies are also applicable as an alternative to ALP staining. With respect to lectin staining with rBC2LCN and immunostaining with anti-TRA-1-60 antibody, it is noteworthy that the background staining of hMSCs was distinctly less than that seen with ALP staining and negligible, although immunostaining with SSEA-4 antibody showed background staining of some hMSCs, possibly due to the expression of SSEA-4 in hMSCs [29]. In fact, the number of hiPSC colonies re-counted after staining with rBC2LCN on ALP-stained dishes was equivalent to or slightly higher than the number counted using ALP-stained dishes, indicating that hiPSC colonies can be counted accurately with these alternative staining methods and methods with less background staining are preferable for colony identification.

PCR-based assay using pluripotency marker genes is also known to be a highly sensitive assay for the detection of residual hPSCs, and the LOD was reported to be 0.002% for qRT-PCR [20] and 0.001% for

ddPCR [21]. In addition, PCR-based assay is faster than HEC assay, which needs a culture period of about 1 week. However, it is known that detectable expression of pluripotency marker genes is retained through the early differentiation process of hiPSC-derived MSCs [23]. This indicates that PCR-based assays using an inappropriate marker gene may inadvertently detect partially differentiated cells, which have no tumorigenicity potential, especially when progenitor cells, instead of fully differentiated cells, are intended as the final product. Thus, HEC assay can complement PCR assay since HEC assay can be used for any CTPs by directly identifying the colony derived from a single hPSC.

A small number of residual undifferentiated hPSCs in CTPs can also be detected by *in vivo* immunodeficient mouse studies [10]. Recently, MEASURE multisite studies revealed that 10–100 undifferentiated hiPSCs caused teratomas when hiPSCs (ChiPSC18) were spiked into 1×10^6 normal human dermal fibroblasts and subcutaneously injected into NOD/SCID/ γ C^{null} mice with Matrigel (Tanaka *et al.*, unpublished observation). Currently, the detection sensitivity of *in vitro* assays (HEC and PCR) is equivalent to or more sensitive than *in vivo* assays, and these assays are expected to detect about 10 residual undifferentiated hPSCs in 1×10^6 CTPs (0.001%). However, a further increase in the sensitivity of these assays is required for detecting undifferentiated hiPSCs and the corresponding types of CTPs. For example, to treat heart failure, it is presumed that approximately 10^9 cardiomyocytes derived from hPSCs need to be transplanted into a patient [30]. Therefore, conventional assays would fail to detect $<10^4$ undifferentiated hPSCs ($<0.001\%$) contained in 10^9 hPSC-derived CTPs even though they can detect 0.001% of residual hPSCs in CTPs. Since the number of cells required for *in vivo* assays is limited by the maximum feasible dose, enhancing the detection sensitivity of *in vitro* assays by increasing the number of cells that can be assayed is highly desirable for mitigating the potential tumorigenicity risk of residual undifferentiated hPSCs in CTPs. With regard to this point, Kono *et al.* [31] recently reported that adenovirus and adeno-associated virus-based cytotoxic vectors, which possess strong cytotoxicity to differentiated cells but not to hPSCs, can concentrate cells expressing undifferentiated cell markers in hiPSC-derived products; thus, the overall LOD of HEC assay could be improved to approximately 0.00003% through this biological tool.

MACS is a common method for separating various cell populations depending on their surface antigens. Moreover, its cell loss is much less than that seen when using fluorescence-activated cell sorting [32]. Therefore, the authors investigated whether the MACS system can also enrich undifferentiated hPSCs by sorting with microbeads of conjugated anti-pluripotent stem cell markers. The efficient sorting of hiPSCs by anti-TRA-1-60 microbeads was confirmed using the ddPCR method. The one-step ddPCR method targeting *LIN28A* transcripts revealed that the numbers of positive droplets (i.e., copy numbers of *LIN28A* messenger RNA) in the eluted fractions were significantly high compared with the flow-through fractions, demonstrating that anti-human TRA-1-60 microbeads were selectively bound and could sort hiPSCs mixed with human T cells. Indeed, 10 (0.00002%) hiPSCs spiked into 5×10^7 human T cells were successfully detected by HEC assay after sorting with anti-human TRA-1-60 microbeads at all three facilities, with high TPR values. The results from present multisite experiments clearly show that the MACS system can enrich targeted undifferentiated hiPSCs by using microbeads of conjugated anti-pluripotent stem cell markers, and by utilizing this system, the detection sensitivity of HEC assay can be improved significantly. The detection sensitivity confirmed by the present experiments is 0.00002%; however, further improvement in sensitivity can also be expected because targeted cells can be positively sorted from a total of 2×10^{10} cells by using commercially available columns according to the manufacturer's instructions. Instead of hMSCs, hPBMC-derived T cells were used for these experiments because hPBMCs were more suitable for expanding the number of cells necessary to evaluate the feasibility of the MACS system. However, the authors' preliminary data showed that hiPSCs could be

enriched using the MACS system (data not shown) even when a small number of hiPSCs were spiked into hMSCs. Though further experimental optimization using other cell types—especially hiPSC derivatives—is necessary, hiPSC enrichment using the MACS system is expected to be applicable for various types of CTPs.

In conclusion, MEASURE multisite experiments using a number of hiPSC lines under various culture conditions were able to confirm the reported LOD (0.001%) of HEC assay and prove its high sensitivity and robustness. In addition, MEASURE experiments demonstrated that enrichment of targeted undifferentiated hPSCs by the MACS system using microbeads of conjugated anti-pluripotent stem cell markers markedly improved the detection sensitivity of this assay (0.00002%). This procedure can also be used with PCR-based assays and is highly useful for mitigating the risk associated with the tumorigenicity potential of hPSC-derived CTPs, especially in cases where a larger number of cells need to be administered clinically. The findings obtained in the present study would contribute to the future standardization of tumorigenicity risk assessment of hPSC-derived CTPs.

Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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Author Contributions

Conception and design of the study: TW, SY and YS. Acquisition of data: SK, TK, MF, MO, HM, EK, HF and MN. Analysis and interpretation of data: TW, SY and YS. Drafting or revising the manuscript: TW, SY, SK, TK, MF, MO, HM, EK, HF, MN and YS. All authors have approved the final article.

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Supplementary materials

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