


Long-term Cryopreservation of Human and other Mammalian Cells at -80°C for 8 Years

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Abstract

Freezing is recognized as the most effective method of maintaining a stable supply of various cell types for long-term storage. However, cells might be damaged by environmental changes during the freezing process. There are various factors that influence the function of cells cultured after cryopreservation and thawing. These factors include cryopreservation solutions, biomaterials, freezing methods, and the freezing and preservation temperatures. There is also a risk of infection with mycoplasma in liquid nitrogen phase. Therefore, it is necessary to consider more useful and safe methods for freezing and storing various cells. In this study, we investigated the effects of temperature during long-term storage (8 years at -80°C and in liquid nitrogen phase) on the quality of various cells (human hepatocellular carcinoma cells, bovine carotid artery normal endothelial cells, mouse fibroblast cells 3T3, and mouse embryo fibroblast cells STO). We examined the cell viability of cryopreserved human hepatocellular carcinoma cells at -80°C using culture medium containing 10% DMSO, Cell Banker 1, and Cell Banker 2 as cryopreservation solutions. Among these solutions, Cell Banker 1 showed the highest efficiency. The viability of human hepatocellular carcinoma and bovine carotid artery normal endothelial cells in the Cell Banker 1 stored at -80°C was over 90%, which was the same as that in liquid nitrogen phase. The cells stored at -80°C had a morphology similar to that of the cells stored at liquid nitrogen phase. The proliferation of cells stored at -80°C and in liquid nitrogen phase was not significantly different. Furthermore, none of the cells were infected with mycoplasma. There was no marked difference in the albumin secretion between the human hepatocellular carcinoma cells stored at -80°C and those in liquid nitrogen phase. The short tandem repeats of the human hepatocellular carcinoma cells stored at -80°C were identical to those stored in liquid nitrogen phase. In this report, various cells stored long-term at -80°C were able to be used effectively after long-term storage. These findings can be applied to drug discovery, cell medicine, and cell therapy.

Keywords

human and mammalian cells, cryopreservation, -80°C , long-term storage, cell quality

Introduction

Freezing for long-term storage has proven to be one of the most effective methods of maintaining a stable supply of various cell types. However, cells may be damaged by environmental changes during the freezing process^{1,2}. There are various factors that influence the function of cells cultured after cryopreservation and thawing, including the cryopreservation solution³⁻⁶, biomaterials^{7,8}, freezing methods^{9,10}, and freezing and preservation temperatures³⁻¹⁰. Among cryopreservation solutions, cryoprotective agents such as glycerol^{11,12}, ethylene glycol¹³, and dimethyl sulfoxide (DMSO)¹⁴ are the most effective due to their high rate of penetration into cells. In addition, it has been reported that starch and oligosaccharides³ like trehalose^{15,16} and maltose^{4,6} are effective in suppressing damage to cells.

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Cells cryopreserved on a collagen thin film⁷ or a carrier material⁸ can be directly applied to transplantation and drug discovery efforts. Freezing methods that reduce cell damage, like the vitrification method, have also been reported^{9,10}. Vitrification is an effective cryopreservation technique of induced pluripotent stem cells (iPSCs)¹⁷ and embryonic stem cells (ESCs)^{9,10}, but the cells become damaged if the osmotic pressure increases. Therefore, more effective and less toxic solutions, as well as more convenient techniques, are strongly desired. Taking into consideration the reduction in cell damage caused by ice crystal formation within the cell and solution troubles such as cell dehydration^{1,2}, it would seem best to store cells in liquid nitrogen (LN₂) phase and the vapor phase of LN₂. However, long-term storage in LN₂ phase carries a risk of mycoplasma infection, bacterial, and viral agents^{18,19}. Therefore, it is necessary to consider more effective methods for freezing and storing various types of cells.

In this study, we investigated the effects of temperature during long-term storage (8 years at -80°C or in LN₂ phase) on the quality of various cells.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and antibiotics (penicillin, streptomycin) were purchased from GIBCO BRL, Life Technologies Inc. (Grand Island, NY, USA). Fetal bovine serum (FBS, BIO-WEST) was obtained from Funakoshi Co., Ltd. (Tokyo, Japan). Dulbecco's phosphate buffered saline without calcium chloride and magnesium chloride (DPBS(-)) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other materials and chemicals not specified above were of the highest grade available.

Cells

HepG2 cells (human hepatocellular carcinoma cells, HB-8065) and STO cells (mouse embryo fibroblast cells, CRL-1503) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HH cells (Bovine carotid artery normal endothelial cells, JCRB0099) and NIH 3T3 cells (Mouse fibroblast cells, clone 5611, JCRB0615) were obtained from the JCRB Cell Bank (Osaka, Japan).

Cryopreservation and Thawing of HepG2 and Mammalian Cells

Cells were cultured on 60-mm culture dishes with 4 mL of culture medium at 37°C in 5% CO₂. The culture medium consists of DMEM supplemented with 10% FBS, 100 U/mL of penicillin, and 100 U/mL of streptomycin. The cells were passaged at least four times and then frozen in a cryopreservation solution. As cryopreservation solutions, the culture medium and 10% DMSO, Cell Banker 1, and Cell Banker

2 (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) were used. One milliliter of a cell suspension containing 1×10^6 cells was quickly transferred to a 2.0-mL cryotube and frozen at a cooling rate of $1^{\circ}\text{C}/\text{min}$. After cooling to -80°C , the cells were stored at -80°C or in LN₂ phase until use (typically over 8 years; HepG2 cells, August 16, 2008; STO cells, September 12, 2008; HH cells, August 8, 2008; and NIH 3T3 cells, August 5, 2006).

The frozen tubes were placed in a water bath at 37°C to thaw until the ice crystals had nearly melted. The cell suspension was then diluted to 1:9 with ice-cold culture medium and centrifuged at 1,000 rpm for 5 min. The supernatant was removed, and the cells were resuspended in fresh medium. The cell viability was assessed using the trypan blue exclusion test. The final concentration of trypan blue (GIBCO BRL; Life Technologies Inc.) was 0.2%.

Culture and Proliferation Assay of HepG2 and Mammalian Cells

After long-term cryopreservation at -80°C , thawed HepG2 cells and mammalian cells (2×10^5 live cells) were seeded into 60-mm culture dishes in 4 mL of the culture medium. The cells were incubated at 37°C under a humidified 5% CO₂ atmosphere, and the culture medium was changed every 24 h. For cell culture, cells were detached from a 60-mm culture dish with trypsin (GIBCO BRL; Life Technologies Inc.) and repeatedly seeded onto a new culture dish.

After 3 to 4 d, the cells attached and spread on 60-mm culture dishes and subcultured until 80% to 90% confluent. After trypsin treatment, cell viability and cell number were determined using a trypan blue exclusion assay. The passages were repeated one to five times. The attached cells were also stained with a kit to evaluate their viability, as described below.

Determination of Living and Dead Cells

The cells were stained with calcein acetoxymethyl ester (calcein AM, 4 mM in anhydrous DMSO) and ethidium homodimer 1 (EthD-1, 2 mM in DMSO/H₂O 1:4 [v/v]; LIVE/DEAD[®] Viability/Cytotoxicity kit, for mammalian cells, L3224; Molecular Probes[®]; Life Technologies Inc.) to visualize living and dead cells. A total of 4 μL of the supplied 2 mM ethidium homodimer-1 (EthD-1) stock solution was transferred to 2 mL of DPBS(-), and then 1 μL of the supplied 4 mM calcein AM stock solution was added to 2 mL of the EthD-1 solution. The working solution containing 2 μM of calcein acetoxymethyl ester (AM) and 4 μM EthD-1 was then added directly to the cells. After the cells had been incubated for 20 min at 37°C , calcein AM-positive green fluorescence in living cells and EthD-1-positive red fluorescence in dead cells were examined using a fluorescence microscope (Keyence Japan, Osaka, Japan).

Mycoplasma Tests of HepG2 Cells and Mammalian Cells

The cells cryopreserved at -80°C and in LN_2 were tested for mycoplasma contamination as below. Cells (2×10^5 cells) were cultured in a 60-mm dish with 4 mL of culture medium. After 48 h of culture, the culture medium was changed, and the cells were incubated for 48 h. Mycoplasma infection in the culture medium after 48 h was assessed using the MycoAlert kit in accordance with the manufacturer's instructions (Lonza Rockland Inc., Rockland, ME, USA).

Quantitation of Albumin Secreted by the HepG2 Cells

HepG2 cells (2×10^5 cells) were cultured in a 60-mm dish, and the culture medium was changed every 24 h. After 48 h of culture, the cells were incubated in culture medium for 24 h. The level of albumin secretion was assessed based on the accumulation of albumin in the culture medium after 24 h using a human albumin enzyme-linked immunosorbent assay (ELISA) quantification kit in accordance with the manufacturer's instructions (Bethyl Laboratories, Inc., Montgomery, TX, USA).

An Analysis of Short Tandem Repeats (STRs) for the Identification of HepG2 Cells

To determine whether the HepG2 cells that had been stored at -80°C were genomically identical to those that had been stored in LN_2 phase, the STRs of the genomes were analyzed^{20,21}. The analysis of the STRs was outsourced to BEX Co., Ltd. (Tokyo, Japan) using the Cell ID System (Promega Corp., Madison, WI, USA).

Results

Cryopreservation of HepG2 Cells in Various Solutions at -80°C

HepG2 cells were frozen and stored at -80°C for 8 years. These cells were cryopreserved in culture medium containing 10% DMSO, Cell Banker 1, and Cell Banker 2. In order to investigate the effects of cryopreservation, we determined the cell viability immediately after thawing (Fig. 1A). The viability of HepG2 cells in the culture medium containing 10% DMSO, Cell Banker 1, and Cell Banker 2 was shown to be over 93% (Fig. 1A). The viability was highest with Cell Banker 1 (96%), so Cell Banker 1 was used in subsequent experiments.

Long-term Cryopreservation of HepG2 and Mammalian Cells at -80°C or in LN_2 phase

HepG2 and mammalian cells were frozen and stored at -80°C or in LN_2 phase for 8 years. These cells were cryopreserved in Cell Banker 1. We investigated the cell functions at cryopreservation temperatures over a long period of time.

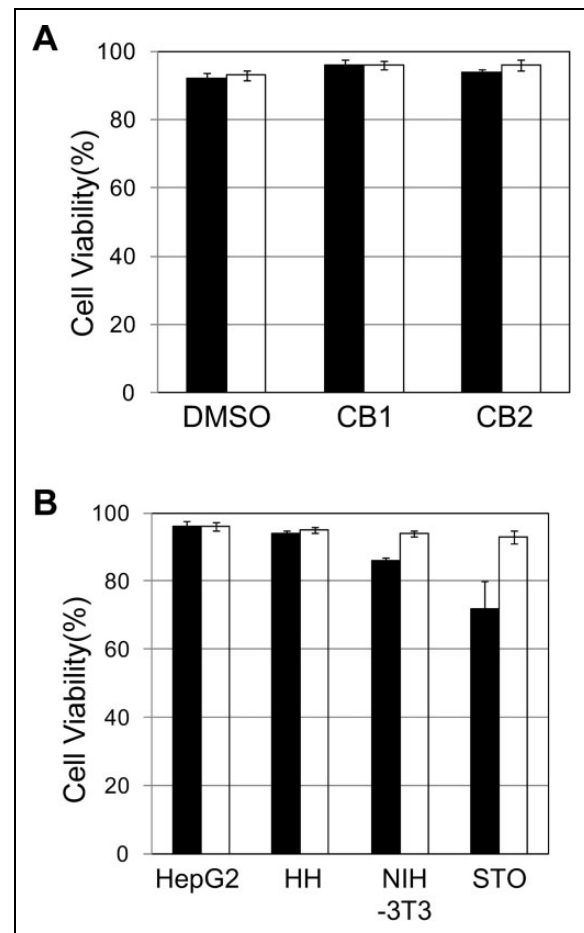


Fig. 1. Cell viability of human and mammalian cells cryopreserved at -80°C and in liquid nitrogen (LN_2) phase for a long period. HepG2, HH, NIH-3T3, and STO cells were frozen and stored at -80°C or in LN_2 phase for 8 years. (A) Cell viability of the cryopreserved HepG2 cells in different cryopreservation solutions. Dimethyl sulfoxide (DMSO): culture medium containing 10% DMSO; CB1: Cell Banker 1; CB2: Cell Banker 2. The cell storage settings were as follows: -80°C (black column), and LN_2 phase (open column). (B) Cell viability of human and mammalian cells cryopreserved with Cell Banker 1. HepG2: HepG2 cells; HH: HH cells; NIH-3T3: NIH-3T3 cells; STO: STO cells. The cell storage settings were as follows: -80°C (black column) and LN_2 phase (open column). The data represent the means and standard deviation (SD) of three independent experiments.

First, we determined the cell viability immediately after thawing (Fig. 1B). The viability of HepG2 cells and HH cells stored at -80°C was over 94% (cell viability of HepG2 and HH cells was 96% and 94%, respectively), which was nearly the same as that of the cells stored in LN_2 phase (cell viability of HepG2 and HH cells was 96% and 95%, respectively). However, the viability of the NIH 3T3 cells and STO cells stored at -80°C (cell viability of NIH 3T3 and STO cells was 86% and 72%, respectively) was lower than that of the cells stored in LN_2 (cell viability of NIH 3T3 and STO cells was 94% and 93%, respectively).

Second, HepG2 cells and mammalian cells stored at -80°C and in LN_2 phase were observed under a

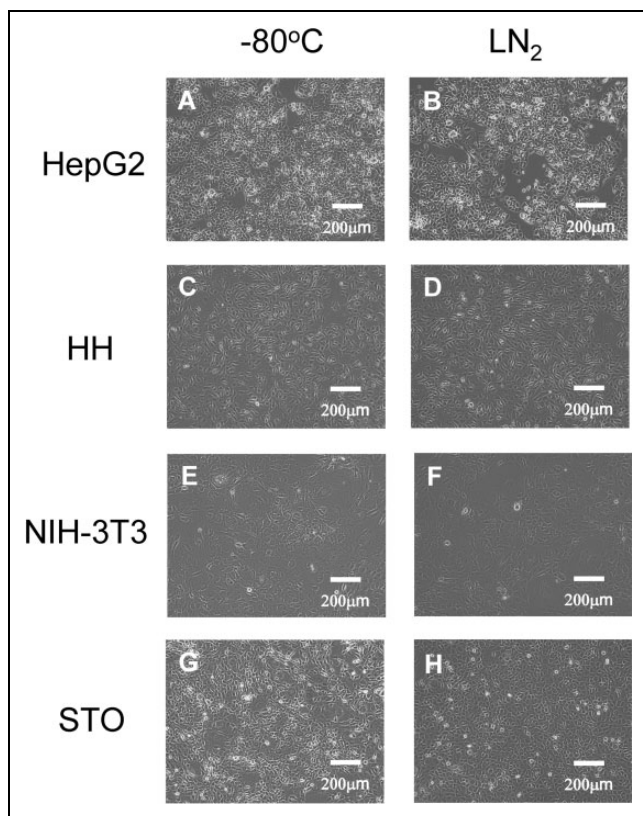


Fig. 2. Phase-contrast photomicrographs of the human and other mammalian cells. HepG2, HH, NIH-3T3, and STO cells at a density of 5×10^5 (A, and B), 2×10^5 (C, and D), 2×10^5 (E, and F), and 2×10^5 cells (G, and H), respectively, were seeded onto 60-mm culture dishes and then cultured for 4 d. The cell storage settings were as follows: -80°C (A, C, E, and G) and liquid nitrogen (LN_2) phase (B, D, F, and H). Scale bar: 200 μm .

phase-contrast microscope (Fig. 2). The morphology of the cells stored at -80°C (Fig. 2A, C, E, and G) was similar to that of the cells stored in LN_2 phase (Fig. 2B, D, F, and H).

Third, after thawing, HepG2 and mammalian cells were stained with calcein AM and EthD-1 to discriminate between living and dead cells (Fig. 3A). Calcein AM-positive green fluorescence in the living cells and EthD-1-positive red fluorescence in the dead cells were observed using a fluorescence microscope. The viability of the HepG2 cells and the mammalian cells stored at -80°C was over 98% (Fig. 3B).

Fourth, to evaluate the proliferation of HepG2 cells and mammalian cells stored at -80°C and in LN_2 phase, we cultured these cells on new culture dishes for 4 d. After cell cryopreservation and thawing, the passages were repeated twice (Fig. 4). The proliferation of HepG2 cells and mammalian cells was not significantly different between those stored at -80°C and those stored in LN_2 phase.

Fifth, the cells cryopreserved at -80°C or in LN_2 phase were tested for mycoplasma contamination (Table 1). After 48 h of culture, the culture medium was changed, and the cells were incubated for 48 h. The results showed that the

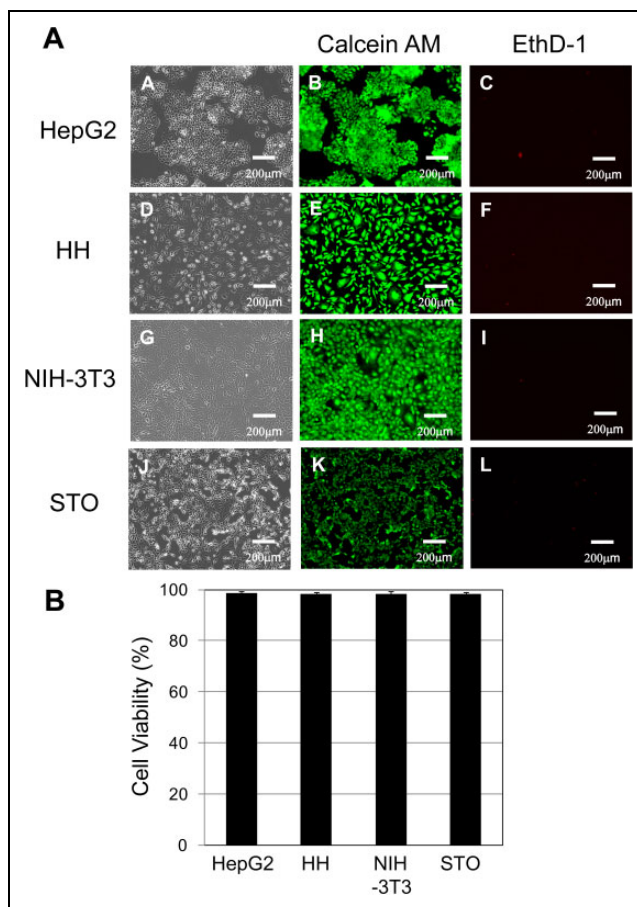


Fig. 3. Cell viability of living/dead cells in the human and mammalian cells. (A) HepG2, HH, NIH-3T3, and STO cells at a density of 2×10^5 cells were inoculated onto 60-mm dishes for 4 d (phase contrast; A, D, G, and J). HepG2, HH, NIH-3T3, and STO cells were stained with calcein acetoxymethyl ester (calcein AM) and ethidium homodimer-1 (EthD-1) to visualize living and dead cells. Calcein AM-positive green fluorescence (B, E, H, and K) in living cells and EthD-1-positive red fluorescence (C, F, I, and L) in dead cells were observed with a fluorescence microscope. Scale bar: 200 μm . (B) Cell viability of human and mammalian cells attached onto 60-mm dishes. HepG2: HepG2 cells; HH: HH cells; NIH-3T3: NIH-3T3 cells; STO: STO cells. The cell storage settings were as follows: -80°C (black column). The data represent the means and SD of three independent experiments.

cultured cells were negative (MycAlertTM ratio of the HepG2 cells and mammalian cells < 0.67) for mycoplasma compared to the positive control (MycAlert ratio of positive control = 95). The cells cryopreserved at -80°C and in LN_2 phase were not infected with mycoplasma.

The Liver Function and STRs of HepG2 Cells at -80°C and in LN_2 phase

We performed a population doublings (PD) test over 1 to 5 passages (Fig. 5A) and measured the quantification of

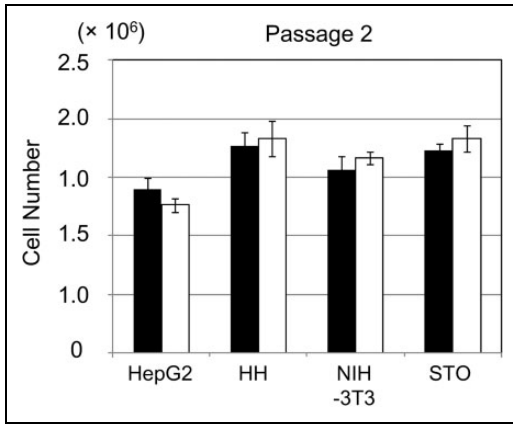


Fig. 4. Proliferation of HepG2 cells and mammalian cells at -80°C and in liquid nitrogen (LN_2) phase. HepG2, HH, NIH-3T3, and STO cells at a density of 2×10^5 cells were seeded onto 60-mm culture dishes and then cultured for 4 d. After cell cryopreservation and thawing, the passages were repeated twice. HepG2: HepG2 cells; HH: HH cells; NIH-3T3: NIH-3T3 cells; STO: STO cells. The cell storage settings were as follows: -80°C (black column) and LN_2 phase (open column). The data represent the means and standard deviation (SD) of three independent experiments.

Table 1. Mycoplasma Tests of Human and other Mammalian Cells at -80°C and in LN_2 Phase for a Long-Term Period.

Cells	Cryopreservation Temperature	MycAlerttm Plus Ratio	Conclusion
HepG2	-80°C	0.45	Negative
HepG2	LN_2	0.65	Negative
HH	-80°C	0.58	Negative
HH	LN_2	0.52	Negative
NIH/3T3	-80°C	0.67	Negative
NIH/3T3	LN_2	0.48	Negative
STO	-80°C	0.61	Negative
STO	LN_2	0.56	Negative
Positive Control		95	
Negative Control		0.17	

Note. The cells cryopreserved at -80°C and in liquid nitrogen (LN_2) phase were tested for mycoplasma contamination as below. Cells (2×10^5 cells) were cultured in a 60-mm dish with 4 mL of culture medium. After 48 h of culture, the culture medium was changed, and the cells were incubated for 48 h. Mycoplasma infection in the culture medium after 48 h was assessed using the MycoAlert kit in accordance with the manufacturer's instructions (Lonza Rockland Inc., Rockland, ME, USA).

albumin secreted by the cells (Fig. 5B) and conducted an STR analysis (Fig. 5C) on the HepG2 cells stored at -80°C or in LN_2 phase. HepG2 cells at 2×10^5 cells were seeded onto 60-mm dishes and then cultured for 4 d (1 passage per day; Fig. 5A). The population doublings of HepG2 cells and mammalian cells were not significantly different between the cells stored at -80°C and those in LN_2 phase (Fig. 5A). To examine the liver function of the HepG2 cells, we also evaluated the amount of albumin secretion in the

medium over 24 h after 3 d of culture (Fig. 5B). There was no marked difference in the albumin secretion of the HepG2 cells stored at -80°C ($1.1 \mu\text{g}/\text{dish}/24 \text{ h}$) and those stored in LN_2 phase ($1.1 \mu\text{g}/\text{dish}/24 \text{ h}$; Fig. 5B). The STRs of the HepG2 cells stored at -80°C and in LN_2 phase were identical, indicating that the cells were derived from the same origin HepG2 cells (ATCC; Fig. 5C).

Discussion

There have been many reports of cells cryopreserved in LN_2 or in other cryopreservation solutions^{3-6,11-16} or with different freezing methods^{9,10}. We previously reported that oligosaccharides³ and sericin⁴ were effective cryoprotectants against primary human hepatocytes with greater freezing damage.

However, few reports^{5,6,22} have described the effects of cryopreservation of human and mammalian cells at -80°C . Although we reported on the cryopreservation of adipose-derived stem cells^{6,22} and iPS cells⁵ for short periods, such as 1 month, the quality of cells cryopreserved for longer periods must also be evaluated. Therefore, in this study, we examined the outcomes of human and mammalian cells frozen and stored at -80°C or in LN_2 phase over 8 years ago.

The viability of HepG2 cells stored at -80°C was evaluated using three types of cryopreservation solutions (Fig. 1A). The Cell Banker 1 solution resulted in the highest viability, so other mammalian cells were also evaluated using this solution. The cell viabilities of HepG2 cells and mammalian cells stored in LN_2 phase were over 93% (Fig. 1B), whereas those of cells stored at -80°C were lower, depending on the type of cell (Fig. 1B). Since the cell viability varies by cell type, it is necessary to optimize the composition of the cell cryopreservation solution. The cells frozen and stored at -80°C and in LN_2 phase were thawed and then cultured, and the survival of the adhered cells was evaluated using calcein AM and EthD-1 (Fig. 3A). All of the adhered cells maintained their original morphology. No marked differences were observed in the cell morphology due to storage temperature. In addition, the cell viability of the adherent cells stored at -80°C was over 98%, indicating that long-term storage at -80°C is effective. Furthermore, the quality of cells passaged frequently was maintained, as there were no differences in the cell proliferation due to long-term storage temperature (Fig. 4).

Infection with mycoplasma was also evaluated in these cryopreserved cells. No mycoplasma infection was observed in any cells stored at -80°C or in LN_2 phase (Table 1). In contrast to LN_2 phase, cells stored at -80°C are kept in a freezing box under a gas phase; as such, the risk of contamination^{18,19} with agents like the mycoplasma, bacteria, and virus agents can be avoided. Therefore, large volumes of cells can be cryopreserved for a long time with easy handling and high safety at a low cost by simply transferring cells into the -80°C freezer.

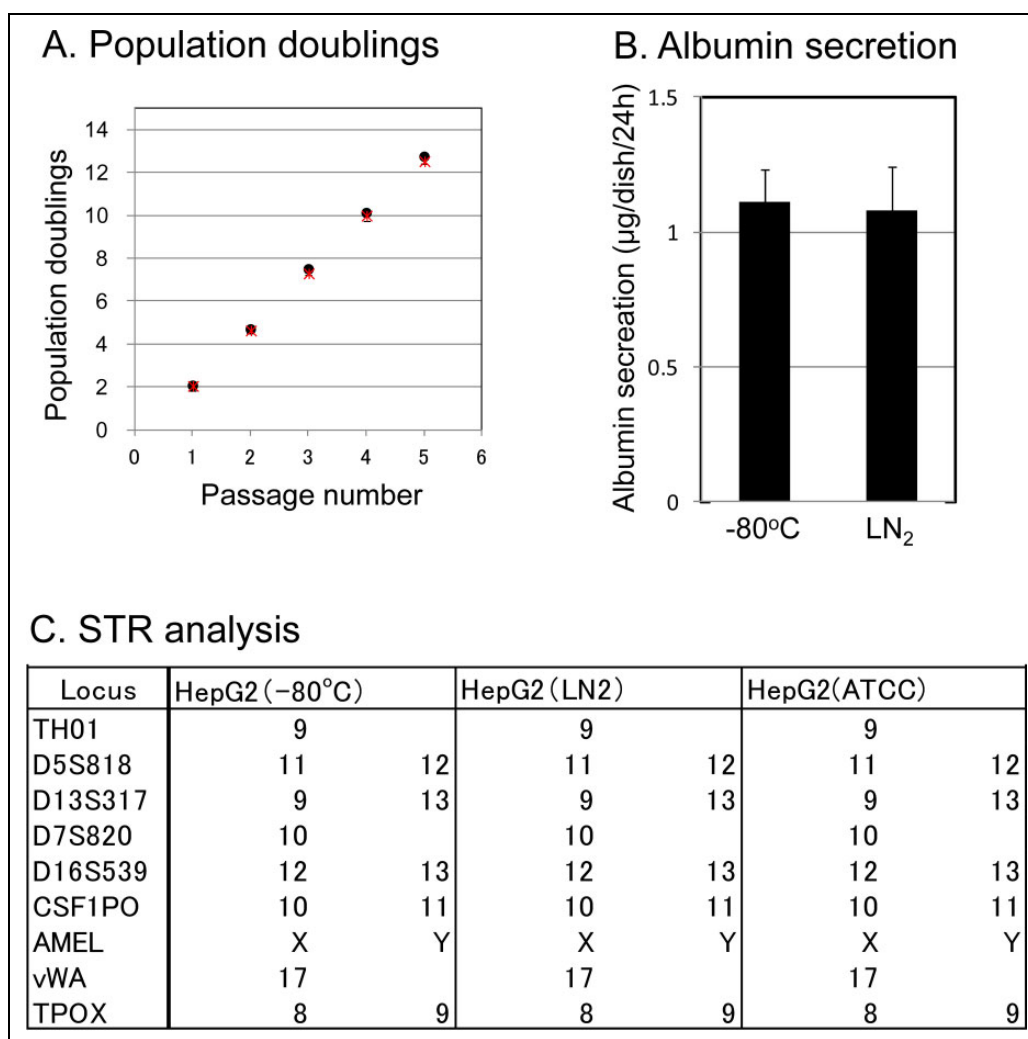


Fig. 5. Population doublings, liver function, and short tandem repeats (STRs) of HepG2 Cells at -80°C and in liquid nitrogen (LN_2) phase. HepG2 cells were frozen and stored at -80°C or in LN_2 for 8 years. The cell storage settings were as follows: -80°C and LN_2 phase. (A) A population doublings (PD) test in the HepG2 cells during 1 to 5 passages. HepG2 cells at a density of 2×10^5 cells were inoculated onto 60-mm dishes for 4 d, and the passages were repeated 1 to 5 times. The cell storage settings were as follows: -80°C (black circle) and LN_2 phase (red asterisk). (B) Albumin secretion in the HepG2 cells at -80°C and in LN_2 phase. We quantified the albumin secreted by HepG2 cells at -80°C and in LN_2 phase. HepG2 cells at 2×10^5 cells were seeded onto 60-mm dishes and then cultured for 3 d. The amount of albumin secreted into the medium was measured by enzyme-linked immunosorbent assay (ELISA) after 24 h of accumulation on day 3 of culture. The data represent the mean \pm standard deviation (SD) of triplicate experiments. (C) The STR analysis to confirm the identity of HepG2 cells. We performed an STR analysis to confirm that the HepG2 cells stored at -80°C and in LN_2 phase were of ATCC origin. The allele data are presented as a table on the right side of each image. The locus indicates the name of the gene analyzed. Genes presented by one datum point were homo and those presented by two data points were hetero.

Finally, we examined the function and conducted an STR analysis of HepG2 cells stored at -80°C and in LN_2 phase (Fig. 5A, B, and C). There were no marked differences in the function of HepG2 cells by cryopreservation setting (-80°C and in LN_2 phase). The STR results confirmed that the cells were equivalent, supporting the effectiveness of frozen storage at -80°C .

In this study, evaluating the efficacy of the preservation of frozen human and mammalian cells not only for the short-term but also for the long-term is extremely important in the fields of medicine and drug discovery. We hope that our findings will be useful for fundamental and clinical

studies using cells. In conclusion, various cells stored for a long time at -80°C were able to be effectively used. These findings are expected to be applicable in drug discovery tests, cell medicine, and cell therapy.

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Ethical Approval

This study was approved by our institutional review board.

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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