Efficient aseptic and automatable vitrification of human pluripotent stem cells using bio-safe and chemically defined media

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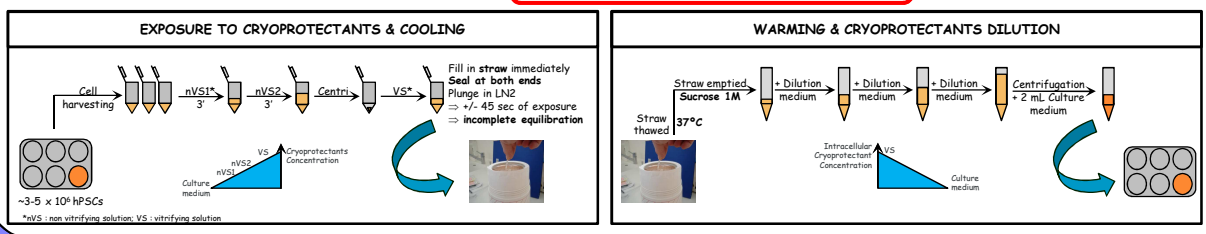
Introduction

Efficient recovery of live and undifferentiated cells is a major challenge in the process of isolating, treating, banking and expanding **human pluripotent stem cells** (hPSCs). **Cryopreservation** is a key step to deal with since cells are submitted to extreme chemical and physical conditions prone to greatly alter their viability as well as their biological properties. Moreover, commonly used **slow freezing (SLF)** and **vitrification**[1] **(VIT)** protocols cannot provide any insurance of biological safety since cells are stored in containers that are predisposed to leakage when plunged into liquid nitrogen (LN2).

We describe our newly developed hPSCs (hESCs [human embryonic SCs – RCM-1 cell line] and hiPSCs [human induced PSCs – dKips & gRips cell lines]) cryopreservation method based on **aseptic VIT** (no direct contact with LN2) using only **chemically defined materials and media**, and amenable to **automation**.

[1]: Vitrification is a cryopreservation method based on the conversion of a liquid into a glass-like state by an infinite enhancement of its viscosity and without formation of ice crystals.

hPSCs Vitrification Protocol



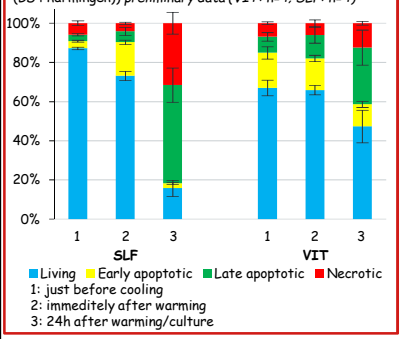
Aseptic vitrification in sealed straws is in accordance with EC and FDA directives on cells and tissues handling and storage stating that cells must be protected from any possible toxic or biological contamination source when intended for therapeutic use.

Results

1. Are aseptic VIT and conventional SLF equivalent for hPSCs cryopreservation?

Assessment of hiPSCs survival rate (dKips)

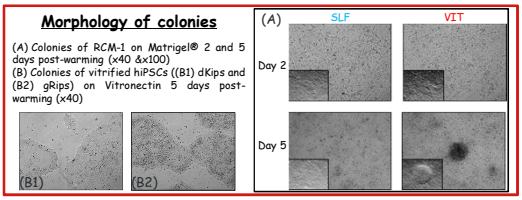
Apoptosis assay (FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen)) *preliminary data (VIT: n=4; SLF: n=4)*



1: just before cooling
2: immediately after warming
3: 24h after warming/culture

Morphology of colonies

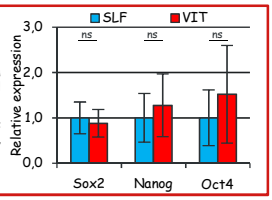
(A) Colonies of RCM-1 on Matrigel® 2 and 5 days post-warming (x40 &x100)
(B) Colonies of vitrified hiPSCs ((B1) dKips and (B2) gRips) on Vitronectin 5 days post-warming (x40)



Gene expression: analysis by qRT-PCR

RNA extracted from each confluent RCM-1 cell culture

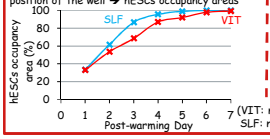
Expression analysis of three major pluripotency transcription factors (VIT: n=13; SLF: n=11)



Morphometric analysis

Cell growth post-warming

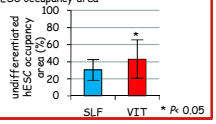
Picture of each culture every day at the same position of the well ➔ hESCs occupancy areas



(VIT: n=22; SLF: n=19)

Ratio of undifferentiated areas

Analysis before cell culture reached confluence ➔ ratio undifferentiated hESC occupancy area/ total hESC occupancy area



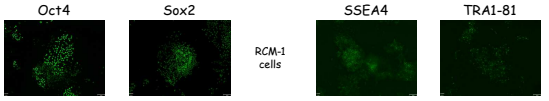
* P< 0.05

Thus, after aseptic VIT or conventional SLF:

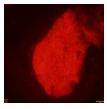
- No major difference among survival rates immediately after warming but 3X more living cells 24h after warming in aseptic VIT conditions
- No obvious difference in terms of cell morphology
- A slight growth delay after VIT but a better maintenance of undifferentiated status
- No significant difference in Sox2, Nanog and Oct4 mRNA expressions

2. Do hPSCs remain cytogenetically stable and pluripotent after aseptic VIT?

Expression of pluripotency markers : analysis by immunofluorescence



Alkaline Phosphatase staining

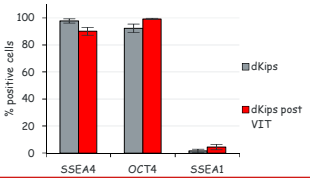


Karyotyping of hESCs

Normal 46XX karyotype

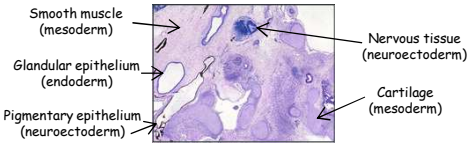
Expression of pluripotency markers : analysis by FACS

Expression analysis of two major pluripotency markers (SSEA4 & OCT4) and a differentiated status marker (SSEA1) of hiPSCs (dKips; n=3)



Assessment of pluripotency status : teratoma formation

Injection of vitrified gRips cells into dorsal flanks of NOD/SCID mice (slides observation after HE staining, X10)



Thus, after aseptic VIT:

- Detection of a majority of Oct4, Sox2, SSEA4 & TRA1-81 positive cells, all pluripotency markers and few differentiated cells
- Positive alkaline phosphatase staining, a marker of undifferentiated status
- Evidence of differentiated elements of the 3 germ layers after teratoma formation
- Observation of a normal 46XX karyotype

Conclusions

We have demonstrated on hPSCs that, despite additional constraints (aseptic, defined medium, automatable…), our aseptic VIT method is more efficient than conventional SLF. We can also conclude that the cells keep their biological (pluripotency) properties after aseptic VIT, including a normal karyotype and the capacity to generate teratoma when injected in immunodeficient mice.

Aseptic VIT of hPSCs is now possible in **completely defined media**. Because straws are directly immersed in LN2, this method does not require any specific and expensive material. To limit cell manipulations, our protocol implies stepwise addition and dilution of cryoprotectants before cooling and after warming, respectively, allowing **automation**.

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