Efficacy of a liquid nitrogen-free electronic controlled rate freezer for the cryopreservation of human mesenchymal stromal/stem cells (MSCs)

Comparison between liquid nitrogen and liquid nitrogen-free controlled-rate freezing for the cryopreservation of MSCs.

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1. Introduction

1.1 Background

Umbilical cord-derived mesenchymal stromal/stem cells (MSCs) have emerged as one of the promising cell therapy products due to their self-renewal, paracrine function, anti-inflammatory, and immunomodulation properties, as well as their ability to differentiate into multilineage cells (1-2). In 1995, Arnold Caplan's team first discovered the therapeutic potential of MSCs (3), and since then many studies have been published on the use of MSCs for clinical trials (1). MSCs for therapeutic applications are classified as advanced therapy medicinal products (ATMPs) and must be manufactured according to current good manufacturing practice (cGMP) guidelines (4). The advancements of various new technologies to produce clinical-grade MSCs *in vitro* on a large scale have paved the way for their use in clinical applications. Cryopreservation and cell banking are key to making off-the-shelf MSC-based ATMPs available (5-6).

Successful cryopreservation is a crucial step for manufacturing and effective clinical delivery of MSCs for therapeutic use and is therefore, an integral part of a commercially robust business model. Crucial steps in the cryopreservation process notably include the selection of cryoprotective medium, freezing equipment, cooling profile, and the cryogenic storage of the cryopreserved product (7). Liquid nitrogen (LN_2) controlled-rate freezers (CRFs) are well established and have been widely used to cryopreserve cellular products (8). However, the use of LN_2 in a controlled environment such as cGMP production areas poses certain challenges. For example, the use of LN_2 can be a source of microbial cross contamination and poses certain serious health risks to operators (9-11). Also, LN_2 cannot be used in cleanrooms where most ATMP manufacturing takes place (Fig 1). An alternative to standard LN_2 operating CRFs has emerged in recent years, such as the VIA Freeze™ instrument from Cytiva. These systems cool samples using conduction cooling, driven by an internal Stirling cryocooler that brings the CRF's chamber to cryogenic temperatures with electricity (12). However, there has been little assessment of LN_2-free CRFs using clinical scale materials.

1.2 Purpose

We evaluated the efficacy of the LN_2-free CRF from Cytiva (VIA Freeze™ Quad system) by comparing it to a classical LN_2 CRF from Planer PLC (Kryo 560) for the cryopreservation of human umbilical cord-derived MSCs. Ultimately, the aim was to determine if the VIA Freeze™ Quad system can be used within NHS Blood and Transplant (NHSBT) to cryopreserve MSCs and to improve NHSBT's services in the future. To assess the quality of the cryopreserved samples, various essential parameters were analysed such as viability and phenotypic characteristics following the International Society for Cellular Therapy (ISCT)'s MSC defining criteria as shown in Table 1 (13).

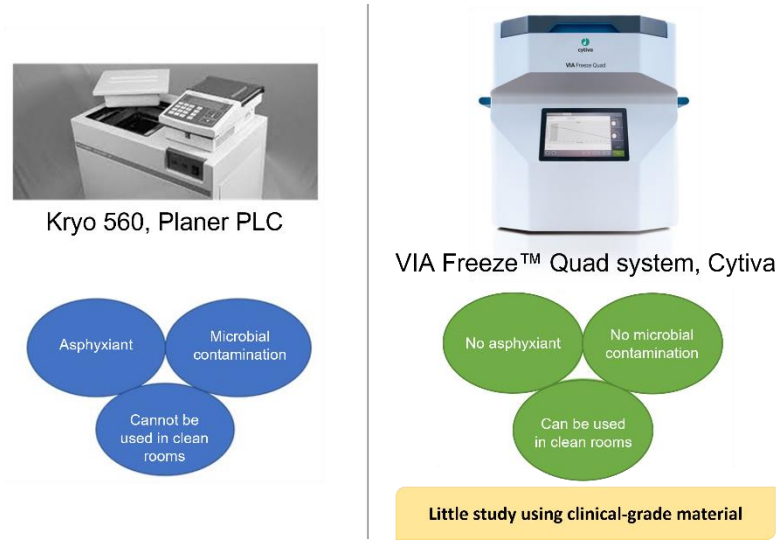

Fig 1. Comparison between LN_2 controlled rate freezing exemplified by the Kryo 560 from Planer PLC versus LN_2-free controlled rate freezing exemplified by the VIA Freeze™ Quad system from Cytiva.

Table 1. Summary of minimal criteria for defining multipotent mesenchymal stromal cells as established by the International Society for Cellular Therapy in 2006.

Criteria #	Description		
1	Adherence to plastic in standard culture conditions		
2	*In vitro* differentitation into osteoblasts, adipocytes, and chondroblasts by means of the colony forming unit (CFU) assay		
3	Phenotype	Positive ≥ 95%	Negative ≤ 2%
		CD105, CD73, and CD90	CD45, CD34 CD14 or CD11b, CD19 or CD79α, and HLA class II

1.3 Overview of the study

Research and development-grade human umbilical cord-derived MSCs were used as starting material. Briefly, samples were mixed with a cryoprotective solution, split into two equal volumes, and transferred to freezing bags; one was cryopreserved in the LN_2 CRF and the other one in the LN_2-free CRF, using set freezing profiles. Pre- and post-thaw analyses were performed to identify any influence of the cryopreservation method.

1.4 Terminology

Term	Comment
ATMP	advanced therapy medicinal product
CCM	complete culture medium
CFU	colony-forming unit
cGMP	current good manufacturing practice
CRF	controlled-rate freezer
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
FGF	fibroblast growth factors

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HLA	human leukocyte antigen
HPL	human platelet lysate
ISCT	International Society for Cellular Therapy
LN_2	liquid nitrogen
MEM	minimum essential medium
MSC	mesenchymal stromal/stem cell
NHSBT	NHS Blood and Transplant
PBS	phosphate buffered saline
SD	standard deviation

2. Material and Methods

2.1 Cell culture

MSCs at passage number three were obtained from the Advanced Therapy Unit, NHSBT-Liverpool and experiments were undertaken between passages four to six. Cells were cultured and expanded in a complete culture medium (CCM) consisting of minimum essential medium (MEM-α) supplemented with fibroblast growth factor-2 (FGF-2) and 10% human platelet lysate (HPL) and were incubated at 37°C in a humidified atmosphere of 5% CO_2, after seeding at a density of 5000 cells/cm^2 in T175 tissue culture flasks. The culture medium was exchanged every two days and cells were harvested at approximately 70% confluency. For passaging and harvest, cells were washed twice with phosphate buffered saline (PBS), before adding 5 mL TrypLE™ Select (Gibco) to the flask and incubating for 5 minutes at 37°C in the humidified atmosphere for enzymatic dissociation, as per manufacturer's instructions. To inactivate the enzymes once cells had fully detached, 10 mL CCM were added to the flask. Cell suspensions were transferred to 25 mL centrifuge tubes and centrifuged at 500 × *g* for 10 minutes at room temperature. The pelleted cells were re-suspended in 5 mL CCM, counted, and subsequently used to seed flasks for further expansion or used for cryopreservation. Cell morphology and confluency were assessed throughout the culture process using an inverted microscope (Nikon-TS100).

2.2 Cell counts and viability

Cell counts and viability were measured after each passage using the NucleoCounter NC-200™ automated cell counter with Via1-cassettes (ChemoMetec). Via1-cassettes contain two types of dyes: acridine orange, which stains all cells to give the total cell counts of a sample and 4',6-diamidino-2-phenylindole (DAPI), which stains dead cells.

2.3 Flow cytometry analysis

The phenotypic characterisation of cells was determined by flow cytometry using a BD FACSLyric™ Clinical Flow Cytometry system (BD Biosciences) and the BD Stemflow™ Human MSC Analysis Kit (BD Biosciences, #562245). Following the manufacturer's instructions, cells were re-suspended in 4.5% human serum albumin to reduce non-specific binding of antibodies at a concentration of 5×10^6 cells/mL. Cells (fresh or thawed) were incubated with antibodies labelled with fluorochromes as

follows: CD90 FITC, CD105 PerCP-Cy5.5, CD73 APC, CD34 PE, CD11bPE, CD45PE, CD45PE, and HLA-DR PE. Isotype-matched antibodies were used as controls. Stained samples were incubated for 15 minutes at room temperature in the dark. Thereafter, 1mL of PBS was added and incubation was continued for another 5 minutes before analysis.

2.4 Sterility testing

Sterility testing of samples was performed using BACT/ALERT® system (bioMérieux) under aerobic and anaerobic conditions. In brief, 2 mL of spent medium was inoculated into each the BACTT/ALERT® bottle and sent to National Bacteriology Laboratory at NHSBT-Manchester for incubation and reading.

2.5 Cryopreservation using LN_2 and LN_2-free controlled-rate freezers

Approximately 5×10^6/mL cells were re-suspended in a pre-chilled freezing medium containing HypoThermosol® (BioLife Solutions) and CryoStor® CS10 (STEMCELL Technologies) at a 1:1 v/v ratio, to achieve a final dimethyl sulfoxide (DMSO) concentration of 5%. Cryoprotected cell suspensions were equally halved and transferred into pre-labelled 50 mL CryoMACS® freezing bags (Miltenyi Biotec, #200-074-400). Bags were fitted inside appropriate metallic cassettes (Thermo Fisher Scientific, #4000610) and were subsequently loaded into the LN_2-CRF (Kryo 560, Planer PLC) and LN_2-free CRF (VIA Freeze™ Quad system with 1000 mL bag adapter, #ASY_30066, Cytiva) for cryopreservation, according to the freezing profiles shown in Table 2. After completion, the cryopreserved samples were transferred to the vapour phase of liquid nitrogen in a 24/7 monitored cryotank (MVE series 1000, Chart Industries) and stored until subjected to post-thaw analyses.

Table 2. Details of the freezing profiles used on the liquid nitrogen controlled rate freezer (LN_2 CRF) versus the LN_2-free CRF (VIA Freeze™ Quad system) for the cryopreservation of MSC samples.

Step	LN_2 CRF freezing profile			LN_2-free CRF freezing profile		
	Cooling rate (°C/min)	Temperature (°C)	Hold time (min)	Cooling rate (°C/min)	Temperature (°C)	Hold time (min)
1		+4	Until samples loaded		+4	Until samples loaded
2	-2	-5		-2	-5	
3	-1	-40		-1	-40	
4	-5	-160		-2	-100	
5			Until samples removed			Until samples removed

2.6 Thawing

Cryopreserved MSC samples were stored in the vapour phase of liquid nitrogen for a minimum of two weeks before thawing and subsequent post-thaw analyses, as follows: samples were removed from storage and immediately fully submerged in a 37°C temperature-controlled water bath (Grant, Thermo Fisher Scientific), where they were gently agitated. Samples were removed from the water

bath once no more visible ice was present and post-thaw analyses were performed immediately thereafter.

2.7 **Statistical analyses**

Cell viability and phenotyping data were plotted in Prism (version 9, GraphPad Software) and are presented thereafter as mean ± standard deviation (SD) from three different samples cryopreserved independently. Statistical analyses were performed in R (version 4.0.3). Due to the very small sample size (n=3), non-parametric tests were applied to compare experimental conditions: Wilcoxon or Kruskal-Wallis's rank sum tests, when two or more distributions were compared, respectively. P-values lower than 0.05 were considered significant.

3. Results

3.1 **Phenotypic characterisation**

Microscopic observation of cells at passage three to six revealed a large elongated flat spindle-shaped fibroblast morphology and cell adherence to plastic (see Appendix 1).

Further, cell surface antigen expression for positive and negative MSC markers was analysed by flow cytometry. The immunophenotypic results were identical for fresh cells from passage three to six (data not shown). When immunophenotypic profiling was performed on cells post-thaw, ≥ 95% of cells expressed the MSC markers (CD90, CD105, and CD73) and ≤ 2% expressed the non-MSC markers (CD34, CD11, CD45, CD45, and HLA-DR). These expression profiles were similar for cells post-thaw and demonstrated no significant difference in CFR modality (p-values ≥ 0.369, Fig 2).

These findings suggest that phenotypic characteristics of the cells post-thaw, including morphology, ability to adhere to plastic, and cell surface antigen expression met the criteria for classification as MSCs, regardless of the type of CRF used.

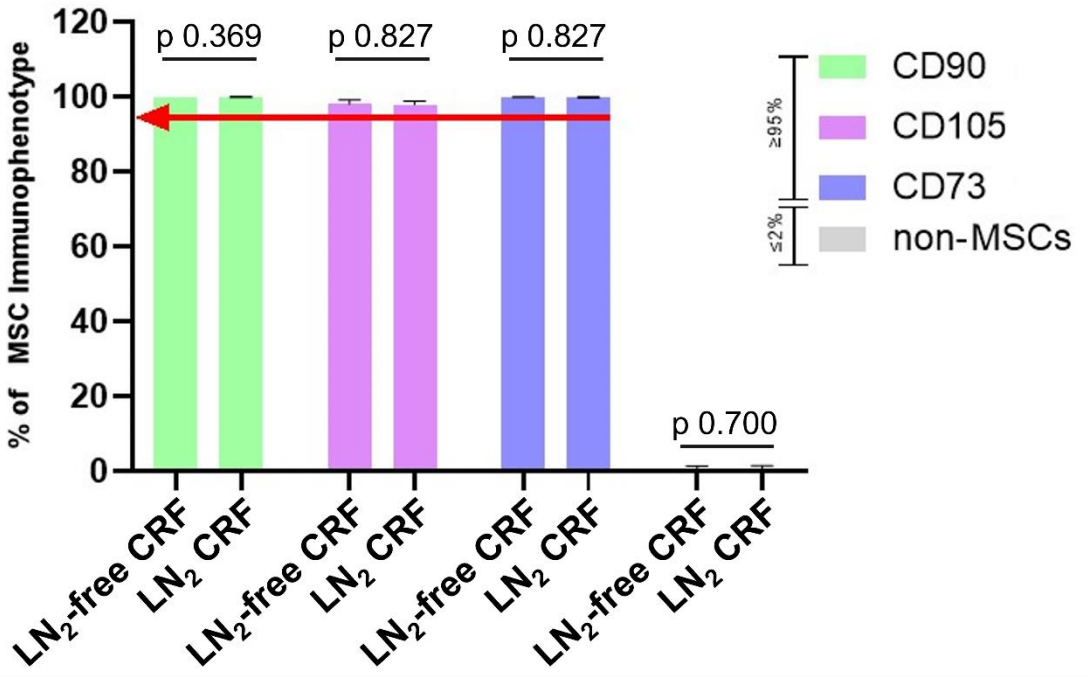

Fig 2. Cell surface antigen expression of cell samples post-thaw to demonstrate the level of MSC purity following cryopreservation in the liquid nitrogen-free controlled rate freezer (LN_2-free CRF) and the LN_2 CRF (n=3; mean ± SD). There was no significant difference between either CRF used (p-values ≥ 0.369). Gating strategy to identify MSCs and non-MSCs in cell populations is shown in Appendix 2. Data provided in triplicate.

3.2 **Cell viability and recovery**

Cell viability pre-freeze was 98.0 ± 1.0%. Cell viability immediately post-thaw was 97.7 ± 1.0% for samples cryopreserved in the LN_2-free CRF and 97.1 ± 1.9% for samples cryopreserved in the LN_2-CRF. No statistical significance was observed between the different cryopreservation methods compared to pre-freeze (i.e., fresh) (p-value = 0.904, Fig 3A). Similarly, post-thaw cell recoveries were 35.1 ± 2.9% (LN_2-free CRF) and 34.2 ± 3.3% (LN_2 CRF) (p-value = 0.747, Fig 3B).

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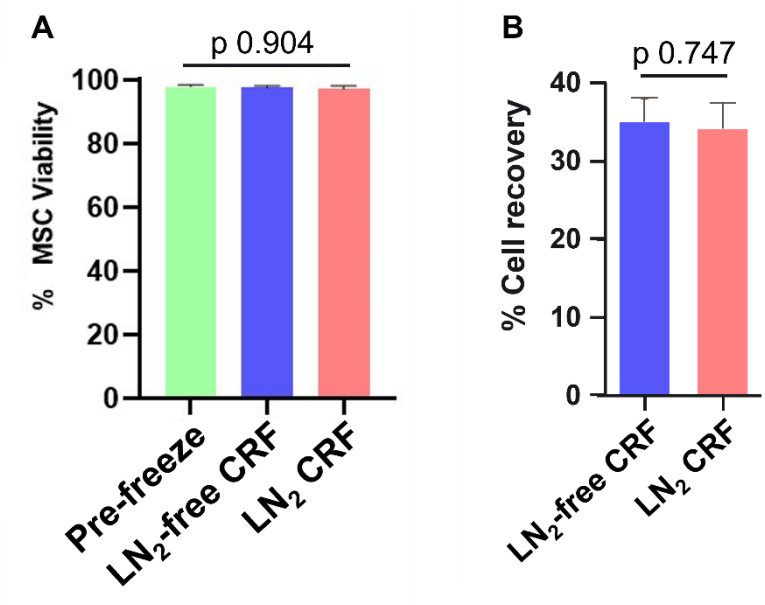

Fig 3. (A) Cell viability pre-freeze and post-thaw, and (B) post-thaw total cell recovery, following cryopreservation in the liquid nitrogen-free controlled rate freezer (LN_2-free CRF) and in the LN_2 CRF. There was no significant difference between experimental conditions (p-values = 0. 904 and 0.747, respectively). Data provided in triplicate (n=3) and shown as means ± SD.

4. Discussion and Conclusion

The concept of off-the-shelf cellular therapy products has become reality with MSC products thanks to three main reasons: their relative ease of manufacturing, manageable ethical concerns (in contrast to, for instance, embryonic stem cells), and most importantly thanks to the fact that allogeneic MSCs can be transplanted without the need for a major histocompatibility complex matching (14). Manufacturing of ATMPs requires adherence to cGMP guidelines as defined in EudraLex Volume 4, Part IV (4, 15). The end-to-end MSC manufacturing process involves cell acquisition (e.g., autologous or allogeneic), expansion, cryopreservation, storage, and shipment for transplantation. During manufacturing, one or more instances of cryopreservation can be performed depending on the culture and expansion protocols in use. Cryopreservation is a key step of the manufacturing process and provides a window for extensive testing of intermediate and/or final products to assess the safety, purity, and potency throughout development before final product release for infusion.

Post-thaw viability is one of the critical factors for clinical applications of MSCs. Success of the cryopreservation process depends on multiple experimental parameters, such as the cryoprotective solution used, the cooling profile applied, and thawing practices. In addition, for clinical application, the CRF should support a cGMP-compliant cryopreservation process. The LN_2-free CRF developed by Cytiva presents the advantage of being ready to integrate with cGMP process validation procedures. This system has successfully been used to cryopreserve a range of clinical scale materials such as peripheral blood mononuclear cells isolated from waste buffy coat and leukocyte cones (16) and

CD34+ cells from umbilical cord blood (17). However, evaluation of the efficacy of LN_2-free CRFs on clinical scale MSC products is lacking.

The LN_2-free CRF developed by Cytiva and used in this study is unique. This system utilizes electricity instead of LN_2 to reach cryogenic temperatures, and therefore presents a number of advantages over standard LN_2 CRFs. Its efficacy to cryopreserve MSC samples was investigated by comparison to a standard LN_2 CRF by measuring cell viability, recovery, and phenotypic characteristics of MSCs, both pre-freeze and post-thaw. Typically, cell viability is expected to be high immediately post-thaw, then viability drops during the first 24 hours of culture post-thaw and increases again in the event of a successful cryopreservation. This transient drop in viability is due to programmed cell death pathways being triggered by damages suffered by cells during the freeze-thaw process (18-19) and has been reported in MSCs (20-22). Our results showed high cell viability immediately post-thaw (≥ 97%) and were expected. These cells also met the viability criteria of the European Medical Agency (Eur Ph. 2.7.29 nucleated cell count and viability) and of the Food and Drug Administration (United States Pharmacopeia cell and gene therapy products) for MSC specifications based on the primary criteria for quality of the final product of ≥ 80% and ≥ 70%, respectively (23-24). In contrast, total cell recovery, showed rather low values (between 34-35% depending on the CRF used). This could indicate that the freeze-thaw process led to extensive damage in a fraction of the cell population such that these cells were destroyed and no longer detectable post-thaw, as reported previously (25). More importantly, no significant differences were observed for either parameter (post-thaw viability and recovery) between both CRFs used.

Phenotypic characteristics, including cell morphology, adherent properties, and cell surface marker expression constitute two of the three minimum criteria proposed by the ISCT to define multipotent MSCs, which our cultured cells met. Previous studies have shown that thawed MSCs maintain fibroblast morphology, adherent properties, and cell surface marker expression of fresh cells (26-27). Our results agreed with previous findings by showing that cells maintained their morphology and surface markers in culture with ≥ 95 % positive expression of MSC cell surface markers (CD105, CD90, and CD73). Most importantly, no difference was seen in the morphology, phenotype, nor viability post-thaw of samples cryopreserved using the LN_2-free CRF or the LN_2 CRF. The biological potential of MSCs to differentiate into trilineage is the third minimal criteria listed by the ISCT to define MSCs (13). The limitation of our study lies in the incomplete understanding of the multilineage differentiation potential of MSCs and its conservation post-thaw.

In conclusion, the data obtained from this study demonstrated that the LN_2-free CRF is a viable option to cryopreserve MSCs and the cells met two out of three standards to fulfil ISCT minimum criteria to identify MSCs. Further analyses would therefore be required to complete the testing, by demonstrating that the cells' multilineage differentiation capability was not either compromised by cryopreservation using the LN_2-free CRF.

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5. Acknowledgments

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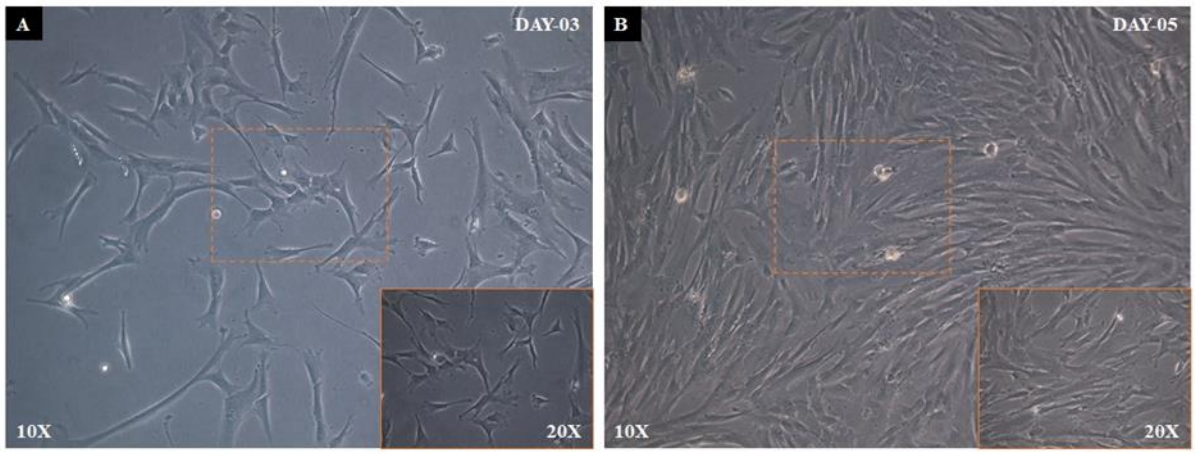
7. Appendices

Appendix 1: Morphology and adherent characteristics of MSCs. Representative images of fresh MSCs captured on (A) day 3 and (B) day 5 of culture showed a fibroblast morphology and adherence to the tissue culture flasks.



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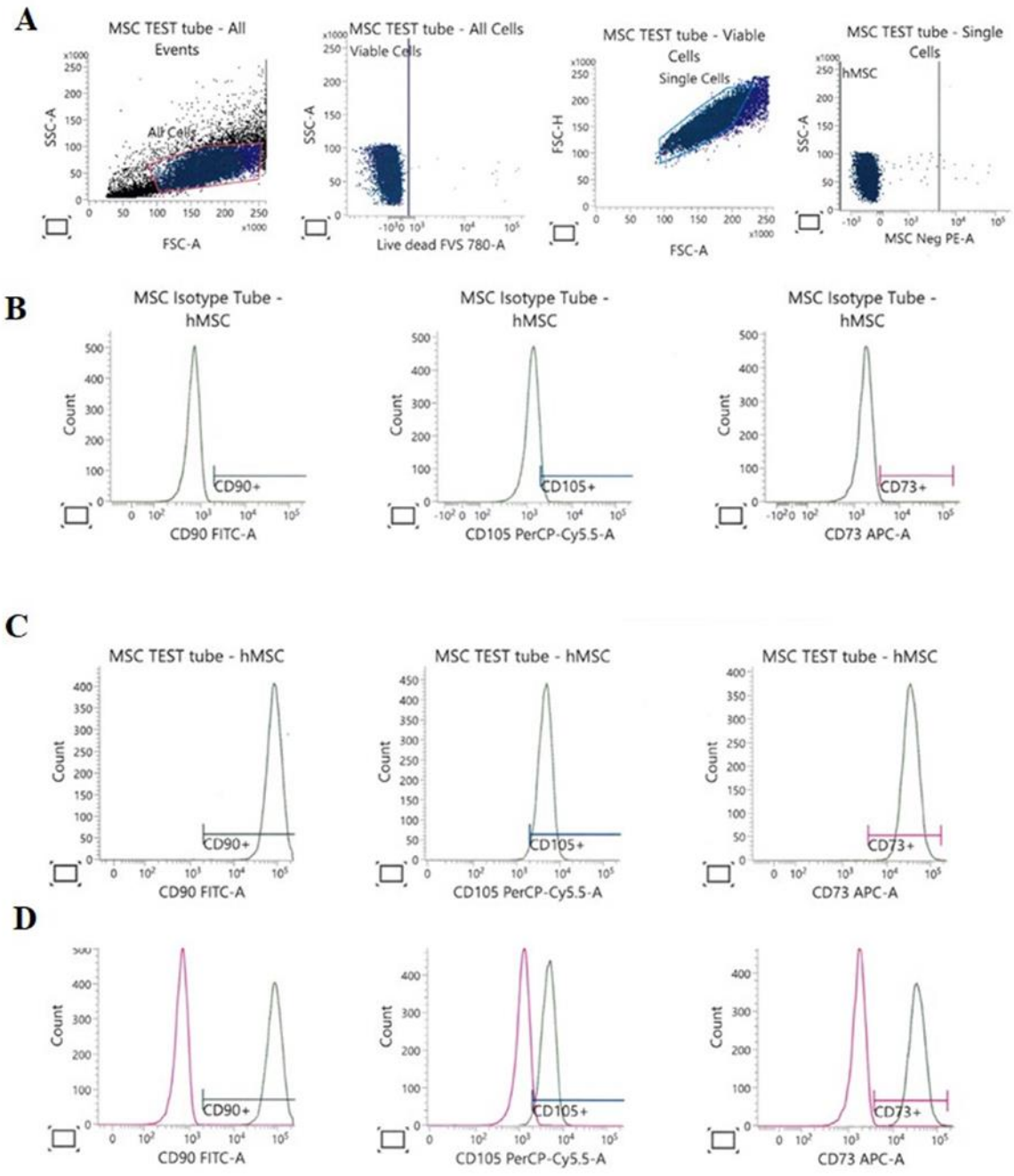
Appendix 2: Gating strategy to identify and evaluate the purity of MSCs. (A) Dot plot to identify MSCs and non-MSCs. Histogram showing (B) negative and (C) positive expression of CD90, CD105, CD73, and (D) overlay of MSCs and non-MSCs cell surface expression.



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