WHITE PAPER

Automated Expansion of Adipose-Derived Mesenchymal Stem Cells (AD-MSCs) with NANT 001 System

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ABSTRACT

The NANT 001 System, designed and manufactured by VivaBioCell, consists in an automated bioreactor and in a single-use sterile cartridge, suitable for cGMP-compliant expansion of both adherent and suspension cells.
The growing demand for Advanced Therapy Medicinal Products (ATMPs) and the challenges owing to their manufacturing require these processes to be standardized, as well as being compliant with Good Manufacturing Practices (GMP) guidelines and Regulatory Agencies requirements, in order to primarily guarantee the safety of these treatments.

This White Paper describes a protocol to automatically expand Adipose-Derived Mesenchymal Stem Cells isolated from fresh adipose tissue (AD-MSCs) using the NANT 001 System.
AD-MSCs obtained by automatic cell expansion with NANT 001 System using a Serum-free cell culture medium - containing human Platelet Lysate supplement (hPL) - were compared to cells manually cultivated using the same medium, and a FBS-based cell culture medium as a control.
Starting from 10 ml of fresh adipose tissue, with the NANT 001 System we were able to obtain $53.5 \pm 16.6 \times 10^6$ AD-MSCs after an average culturing time of 10 days, with over 90% of viability and compliant with the ISCT guidelines for morphology, phenotype and expression of surface markers. Results obtained by a second, independent working group were fully comparable, in terms of quantity, quality and viability of the cells harvested.
Altogether these results demonstrate that automated cell expansion performed with the NANT 001 System meets all the GMP requirements in terms of safety, traceability, reproducibility and reliability, ensuring a more standardized and efficient expansion of AD-MSCs with respect to manual culture procedure.

INTRODUCTION

Mesenchymal stem cells (MSCs) are the spindle-shaped, plastic-adherent cells isolated from bone marrow, adipose tissue and other tissue sources, with multipotent in vitro differentiation capacity. While there is widespread interest in the use of MSCs in a variety of disease targets, to date the processing technology for MSCs isolation and expansion is still focused on traditional, manual and open-circuit protocols. Therefore, there is a strong need to standardize these processes, with the final aim of making the manufacturing of ATMPs not only safer, but also scalable and cost-effective.

The NANT 001 System is a functionally closed and automated system designed to expand both adherent and suspension cells. It evolves from manual cell culture principles, but it is developed to overcome the challenges related to manual resource-consuming processes, with the objective to control and minimize most of the possible risks deriving from operator-dependent open procedures.

The NANT 001 bioreactor (Fig. 1) is composed by a thermostatic compartment in which real-time temperature management is controlled by temperature probes that allow the cell culture to be maintained at a constant temperature of 37°C, a gas flow control system (working with an external Air/CO_2 mixer) that imports CO_2 directly into the cell culture flask to maintain cells at 5% CO_2, a moving tray were the flask is housed, allowing custom movements for optimal washing, seeding and detaching cells procedures. Different movements are possible, including tilting and rocking with customized speed, angle and duration, according to a pre-determined protocol.

Moreover, the NANT 001 bioreactor is equipped with a color-based pH estimation sensor that works with cell culture media containing Phenol Red, as well as with an integrated microscopic imaging system enabling the visual monitoring of the cells, whose confluency is also estimated thanks to a specific integrated algorithm.

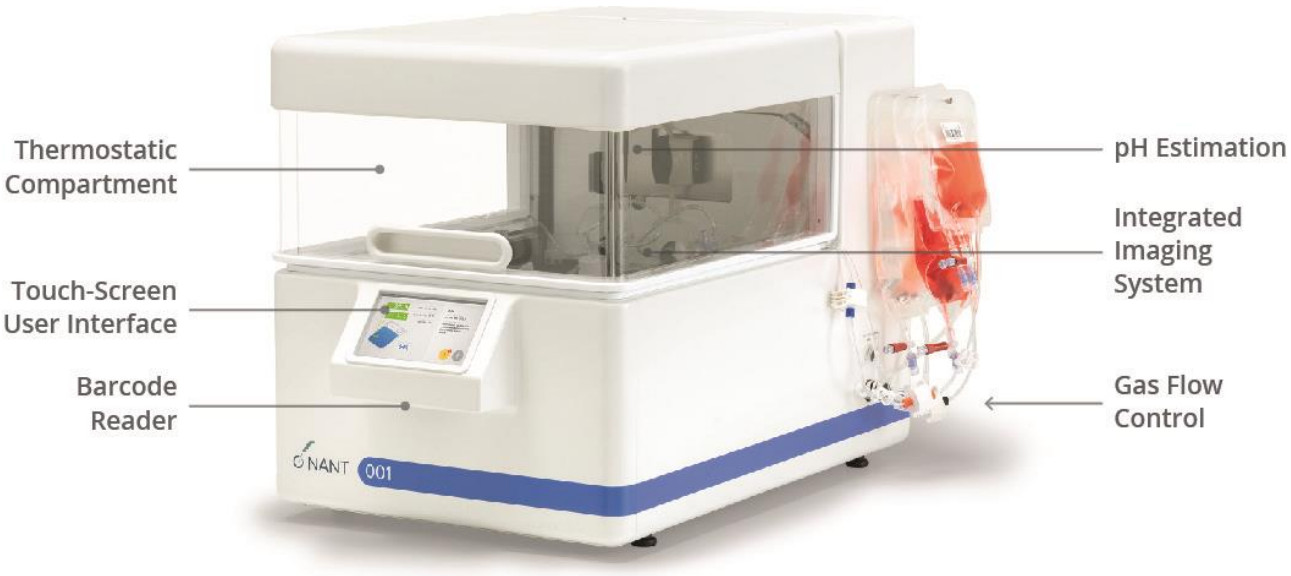

***Figure 1**. NANT 001 Bioreactor exterior view and its main components.*

The NANT 001 Cartridge (Fig. 2) is a sterile, disposable closed system, designed to prevent any contact with the external environment during operations, and equipped with filters and sterile connectors thus allowing the System to be run safely in a Grade D Clean Room. The Cartridge is composed of four different sections (A, B, C and D) that can be quickly and easily connected, when required, thanks to the aseptic connectors; these easy-to-use genderless connectors simplify system integration and minimize the risk of operators' error, without the need for fixtures or tube welders.

The seeding and proliferation surface consists of a tissue culture-treated 636 cm^2 flask, with non-cytotoxic, biologically inert, and nondegradable surface. This flask is ideal for cell culture scale-up, being a useful alternative to roller bottles or spinner flasks, and is suitable for the integration with an imaging system. In order to ensure consistency and reproducibility in even reagent distribution, cell seeding and detachment, the tray hosting the flask can be automatically moved mimicking the manual movements required to optimally perform those operations.

At the end of the expansion process, and after harvesting, cells are collected in a 250 ml Harvesting Bottle which can be easily removed from the rest of the circuit without compromising the sterility, thanks to an aseptic disconnector that prevents external material/contamination from entering into the media flow path upon disconnection. The disconnector's automatic shutoff valves close off the flow path aseptically protecting valuable media while also eliminating the need for pinch clamps and tube welders. Furthermore, thanks to the design of the Harvesting Bottle, the collected cells can be easily centrifuged for downstream processing after replacing the sterile disconnector with another supplied cap.

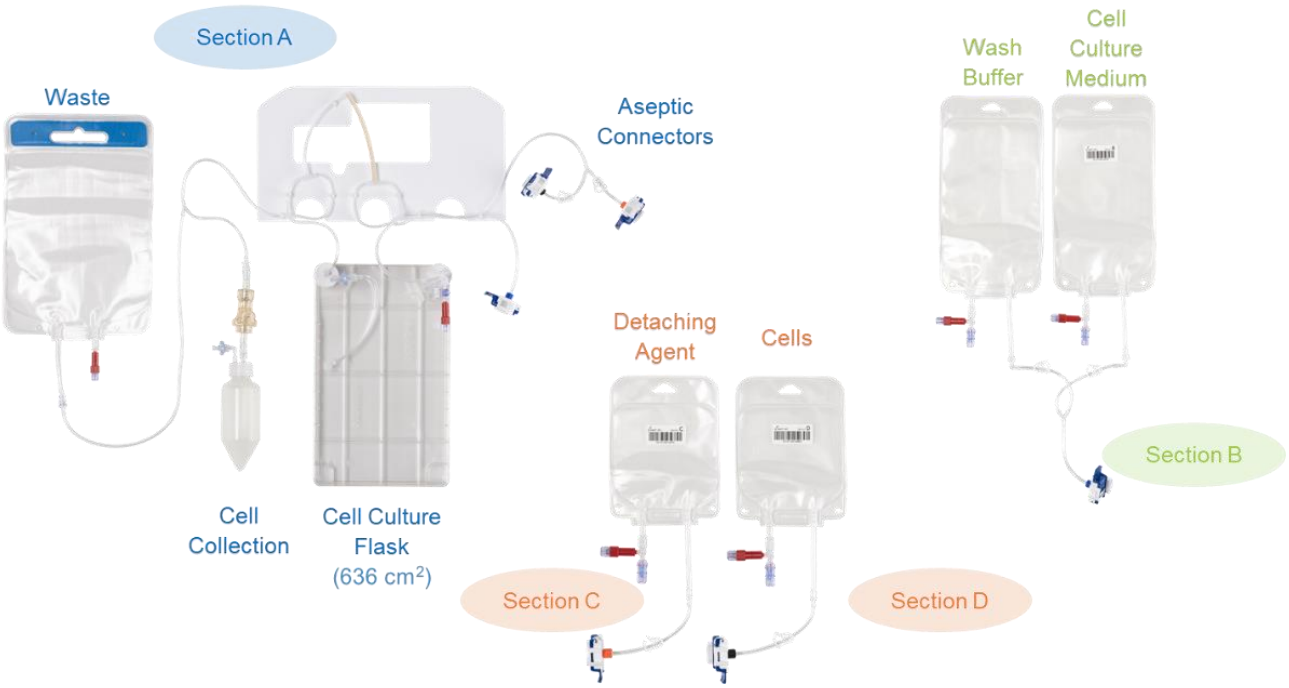

***Figure 2**. NANT 001 Cartridge, the single-use, sterile, disposable unit of NANT 001 System.*

Each section of NANT 001 Cartridge (e.g. cell culture flask, Waste bag, CCM bag, etc.) is equipped with a needle-free one-way sampling port, in order to allow sampling of a desired amount of fluid for any in-process quantitative or quality control analysis, without affecting sterility of the rest of the cartridge.

The use of NANT 001 System automates seeding, expansion and harvesting of a cell culture, allowing for a seamless translation of a manual protocol into a sequence of self-operating phases. All the phases of an automated protocol can be tailored according to a specific process, that is created and uploaded in the bioreactor thanks to a web-based application. In particular, all import and export of fluids, tray position/movements, temperature and CO_2 setpoints, as well as frequency of microscopic imaging acquisition and pH estimation, can be customized during the design of an automated protocol.

A pre-processed input material, such as the Stromal Vascular Fraction (SVF), can be seeded automatically into the cell culture flask and all the cell expansion procedures including washes, media changes as well as the harvesting, are automatically performed by the NANT 001 System according to the pre-determined protocol. The final product is a cell suspension collected in a harvesting bottle, which can be centrifuged and used for further down-stream manipulations.

During the expansion process, cells can be visually monitored thanks to the built-in, liquid lens-based, autofocusing microscope, which periodically provides images of different fields in the flask. An embedded algorithm is able to provide an accurate estimation of cell confluency, allowing for a reliable monitoring of the cell expansion. All the in-process control parameters can be remotely monitored thanks to a dedicated Web-based application, thus reducing the need for accessing into the Clean Room to visually check the progression of the cell expansion; events and warnings occurred during the culture are also accessible from the same interface.

One of the key features prompting the compliance of processes carried out in the NANT 001 System with GMP requirements is the accurate traceability of users, disposables and operations. Indeed, each user and each component of the NANT 001 Cartridge are identified by a unique barcode. Eventually, at the end of the cell culture process, a Cell Culture Report summarizing all the events occurred during the expansion process is automatically generated in an auditable .PDF format file, and is easily downloadable from the same Web-based application.

In this study, an automated protocol for AD-MSCs expansion that utilizes 5% human Platelet Lysate (hPL)-based cell culture medium, was set up. A number of cell expansion experiments were performed with NANT 001 System and compared with manually cultivated cells in the same medium or in a FBS supplemented medium, by two different independent Work Groups.

MATERIALS AND METHODS

The following materials and reagents were used for this study:

- NANT 001 Bioreactor, VivaBioCell, cat.# N1B-01.
- NANT 001 Cartridge, VivaBioCell, cat.# N1C-01.
- T75 flask tissue culture treated, Eppendorf, cat.# 0030711114.
- Phosphate Buffer Saline (PBS), Gibco, cat.# 20012068.
- TrypLE CTS™ (without phenol red), Gibco, cat.# A1285901.
- Collagenase NB4, NordMark, cat.# 17454.01.
- αMEM 5% hPL cell culture medium: αMEM, GlutaMAX™ without nucleosides, Gibco cat.# 32561029; 5% PLTMax , Millipore, cat.# SCM141; 1U/ml Heparin sodium salt from porcine intestinal mucosa, Sigma, cat.# H3393-10KU; 1% v/v Penicillin/streptomycin, Gibco, cat.# 15140122.
- αMEM 4F cell culture medium: αMEM, GlutaMAX™ without nucleosides , Gibco cat.# 32561029; 1% v/v Penicillin/streptomycin, Gibco, cat.# 15140122; 10% FBS of Australian origin, Gibco, cat.# 10099141;10µg/mL Insulin Solution human, Sigma, cat.# I9278; 200 nM Hydrocortisone solution, Sigma, cat.# H6909-10ML; 100 µM pharma grade Vitamin C injectable solution, S.A.L.F.; 10ng/ml hEGF Sigma cat.# E9644-2MG.
- High Yield Lyse fixative-free lysing solution, Invitrogen, cat.# HYL250.
- For cell count: Trypan Blue solution, 0.4%, Sigma cat.# T8154; Countess™ Cell Counting chamber Slides, Invitrogen, cat.# C10228.
- For cytofluorimetric analysis: PE anti-human CD166, eBioScience, cat.# 12-1668-41; APC-eFluor anti-human CD34, eBioScience, cat.# 47-0349-41; PE anti-human CD73 eBioScience, cat.# 12-0739-41; Alexa Fluor 488 anti-human CD31, Thermo Fisher scientific, cat.# MA518135; PerCP-Cy5.5 Mouse anti-human CD105, BD bioscience, cat.# 560819; APC Mouse anti-human HLA-DR, BD bioscience, cat.# 560744; FITC Mouse anti human CD45, BD bioscience, cat.# 555482; FITC Mouse anti human CD14, BD bioscience, cat.# 555397; Propidium iodide, Invitrogen, cat.# P1304MP; ; FACS buffer: Phosphate Buffer Saline (PBS), Gibco, cat.# 20012068 +1% FBS of Australian origin, Gibco, cat.# 10099141, 0.1% Sodium azide, Sigma Aldrich cat.# S8032).

Isolation of SVF from fresh adipose tissue

The adipose tissue used for the experiments described in this White Paper was obtained by Prof. Pier Camillo Parodi, MD (Plastic Surgery Department, Azienda Sanitaria Universitaria Friuli Centrale, Udine, Italy) in the context of lipofilling surgical procedures. Samples were collected following informed consent of patients.

To isolate the SVF, 10 ml of PBS were added to 10 ml of fresh adipose tissue and the sample was centrifuged at 400 × g for 4 minutes. After the centrifugation, the two layers containing mature adipocytes and red blood cells were discarded. The adipose tissue was then incubated with 10 ml of Collagenase NB4 (0.3 PZ U/ml) for 45 minutes at 37°C in agitation. When the digestion was completed, the action of collagenase was inhibited with 10 ml of Cell Culture Medium (CCM) used for cell expansion (αMEM 5% hPL or αMEM 4F) and the mixture was centrifuged for 10 minutes at 600 × g. Finally, the supernatant was discarded and the pelleted SVF was resuspended in 10 ml of CCM.

SVF cell count

1 ml of resuspended SVF was added to 1 ml of High Yield Lyse Buffer and the sample was incubated for 10 minutes at RT in the dark. After the incubation, the sample was centrifuged for 5 minutes at 300 × g and resuspended in 1 ml of CCM. 100 µl of cell suspension were added to 100 µl of Trypan blue 0.4% and counted using a hemocytometer.

SVF flow cytometry analysis.

2 ml of resuspended SVF were incubated with 2 ml of High Yield Lyse Buffer for 10 minutes at RT in the dark. After the incubation, the sample was centrifuged 5 minutes at 300 × g and resuspended in FACS buffer to obtain a concentration of 5 × 10^6 MNCs/ml. 100 µl of cell suspension were incubated with the appropriate set of antibodies and analyzed according to manufacturer's instructions.

Automated cell expansion protocol, and manual control

- 3000 cells/cm^2 were seeded by Work Group 1 in NANT 001 Cartridge with αMEM 5% hPL, or in T75 flask with αMEM 5% hPL or αMEM 4F as manual controls;
- 4000 cells/cm^2 were seeded by Work Group 2 in NANT 001 Cartridge with αMEM 5% hPL, or in T75 or in T225 flask with αMEM 5% hPL as manual control.

NANT 001 automated protocol for AD-MSCs expansion is divided into twelve main different phases, summarized and briefly described in Table 1. Cell expansion in manual controls was performed following the same phases as NANT 001 automated protocol, so that cells were manually processed (washed, subjected to medium changes, and harvested) at the same time as for the automated protocol, and maintained in traditional incubators. For both manual and automated protocol cells were maintained at 37°C and 5% CO_2.

Table 1. *Workflow and description of automated protocol phases.*

Phase #	Phase	Description
1	System initialization	Reaching of temperature and CO_2 settings. Scan and loading of cartridge section A, B (containing CCM and PBS) and C (containing collagenase as a detaching agent).
2	Seeding preparation	Scan and loading cartridge section D (containing cells to be seeded)
3	24h seeding	Cells are distributed and left to adhere to flask surface for 24h
4	Wash and CCM change	24h after seeding, a wash and a full cell culture medium change are performed.
5	Initial expansion and periodic imaging	Imaging and pH estimation are performed every 8h for 48 hours.
6	Wash and CCM change	After 48h of expansion, a wash and cell culture medium change are performed.
7	Expansion and periodic imaging	Imaging and confluency estimation, as well as pH estimation, are performed every 8h until cell confluency reaches 50%.
8	Wash and CCM change	When 50% confluency is reached, a wash and full cell culture medium change are performed.
9	Expansion and periodic imaging	Imaging and confluency estimation, as well as pH estimation, are performed every 8h until cell confluency reaches 90%. After 90% confluency is reached, cells are grown for 24 additional hours.
10	Wash	A wash is performed before import of detaching agent.
11	Detaching and harvesting	Cells are incubated with the detaching agent for 8 minutes. After incubation CCM is added and cells are harvested with specific pre-set movements of the flask.
12	Exporting cells	Cells are collected in the 250 ml harvesting bottle.

The harvesting bottle containing the harvested cell suspension is finally disconnected while maintaining sterility from the rest of the cartridge, and centrifuged for further downstream operations such as washing, concentration and formulation.

AD-MSCs flow cytometry analysis

1.8 × 10^6 cells were centrifuged for 5 minutes at 300 × g and resuspended in an appropriate volume of FACS buffer to obtain a concentration of 3 x 10^6 cells/ml. 100 µl of cell suspension were incubated with the appropriate set of antibodies and analyzed according to manufacturer's instructions.

RESULTS

SVF Characterization

Samples of freshly isolated adipose tissue (about 10 ml of lipoaspirate) were processed by enzymatic digestion to isolate the Stromal Vascular Fraction (SVF). For cells cultivated by Work Group 1, all the SVFs analyzed, displayed an excellent viability (>90%, mean 96.2%; Table 2) and the number of mononuclear cells (MNCs) obtained was comparable in all samples except one (Table 2, Sample 4). The cytofluorimetric analysis revealed high variability in the expression of MSCs surface markers between samples: CD45, CD34, CD14, CD31, CD73 and CD90 (Table 2 and Fig. 3 and 4). CD34, CD73 and CD90 positivity was particularly low in Sample 4, probably due to the low number of MNCs isolated.

Table 2. *Summary of cells isolated by Work Group 1.*

	Volume of tissue processed	No. of MNCs obtained	Viability (Propidium Iodide) (%)	CD34+ CD90+ CD73+ (%)
Sample #1	10 ml	1.36 × 10^7	93.2%	14.3%
Sample #2	10 ml	9.45 × 10^6	98.4%	9.9%
Sample #3	10 ml	1.36 × 10^7	93.9%	7.2%
Sample #4	10 ml	2.68 × 10^6	99.6%	3.2%

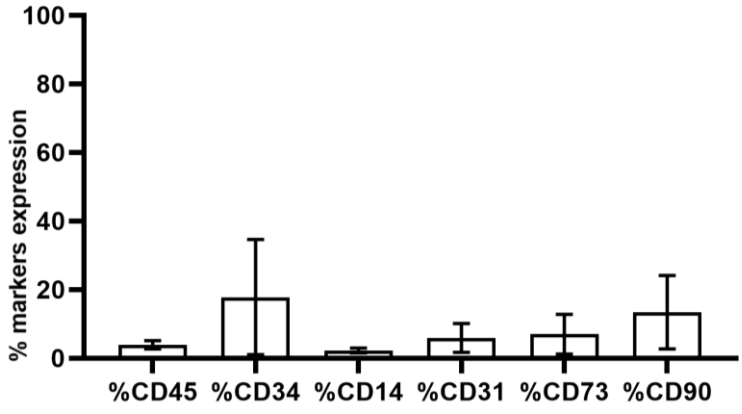

Figure 3*. Average % of surface markers expression in freshly isolated SVF (n=4) obtained by Work Group 1.*

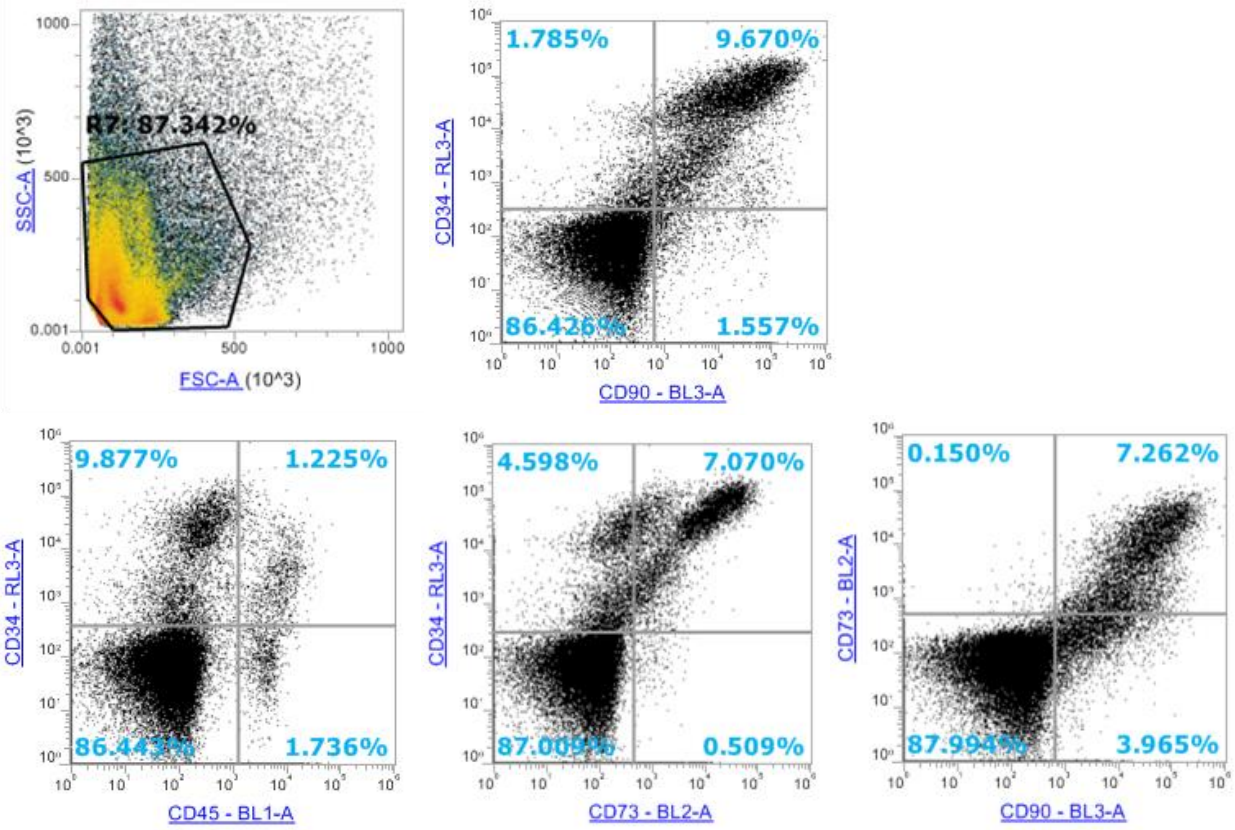

***Figure 4**. Representative example of SVF cells analysis. Morphology and subpopulations were assessed by flow cytometry.*

Viability of the SVF cells resulted very high (mean: 78.7%) also for samples processed by Work Group 2 and, although higher in percentage, the expression of surface markers in the SVF displayed the same high variability (Fig. 5).

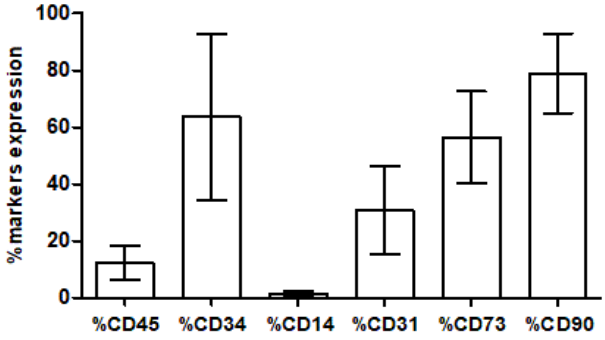

***Figure 5**. Average % of surface markers expression in freshly isolated SVF (n=4) obtained by Work Group 2.*

Automated cell expansion vs manual cell expansion

Both cells cultured manually and with NANT 001 System reflected the morphological characteristics of spindle-like shape (Fig. 6), and both the automated and the manual cultures reached the confluency in 10 ± 3 days. Our results, in accordance with literature (4), confirm that cells grown in media containing hPL revealed a more accentuated spindle-shaped fibroblast-like morphology (Fig. 6).

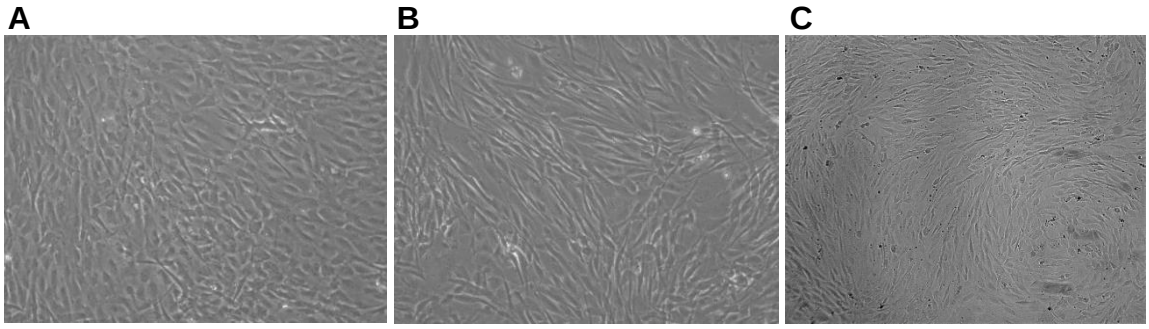

***Figure 6. A**. Confluent cells expanded in αMEM 4F medium using manual protocol (10x magnification); **B**. Confluent cells expanded in αMEM 5% hPL medium using manual protocol; **C**. Confluent cells expanded in αMEM 5% hPL medium in NANT 001 system.*

The average number of cells harvested from NANT 001 in experiments performed by Work Group 1 was 53.7± 16.6 × 10^6 (n=4). Cell density, expressed in cells per square centimeter of the different growth surfaces, was comparable in NANT 001 and in manual controls (Table 3 and Fig.7A; n.s.). Moreover, cells cultivated in NANT 001 System displayed a high and comparable viability (>90%) similarly to cells manually expanded (Fig. 7B). Results from expansions conducted by Work Group 2 were very similar, with an average number of harvested cells of 51.1±12.4 × 10^6 (n=4) (Table 3). The viability of the cells expanded with the NANT 001 System by Work Group 2 was high as well (>90%), similarly to the cells expanded manually.

These data confirm eventually that the NANT 001 System is able to efficiently expand MSCs, with an expected yield comparable - or even slightly higher – than the manual process.

***Table 3.** Summary of the results obtained by Work Group 1 and Work Group 2 in terms of yields, with both NANT 001 System and manual processes.*

	Cell Density (No. of harvested cells/cm^2)	
	Work Group 1	**Work Group 2**
NANT 001, αMEM 5% hPL	8.98 ± 2.29 × 10^4	8.04 ± 1.94 × 10^4
Manual process, αMEM 5% hPL	6.31 ± 1.96 × 10^4	4.29 ± 0.70 × 10^4
Manual process, αMEM 4F	7.32 ± 2.96 × 10^4	-

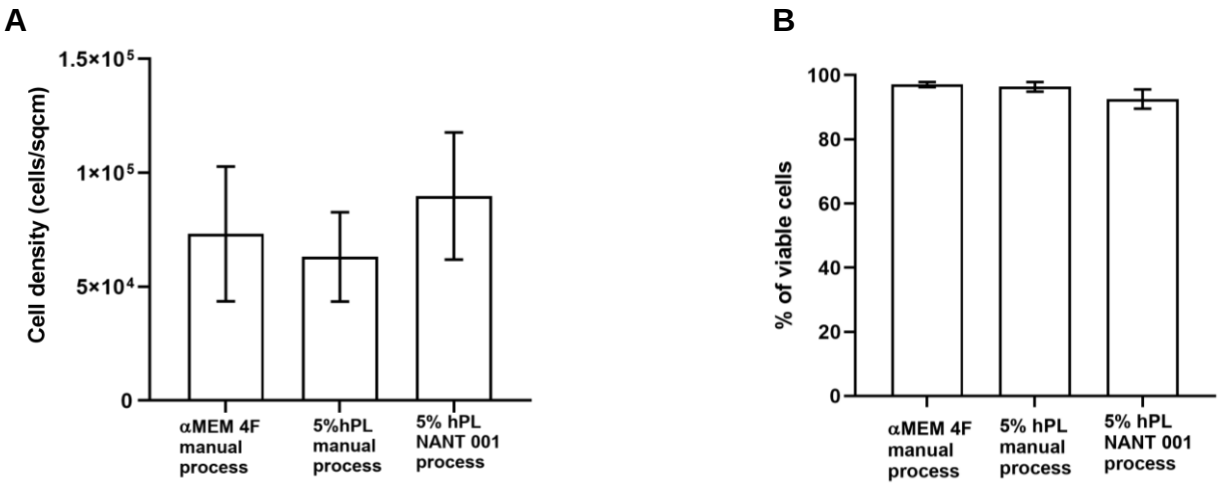

***Figure 7.** Cell density and viability results obtained by Work Group 1; **A**. Number of harvested cells/cm^2 expanded with manual process and NANT 001; B. Viability of cell expanded with manual process and NANT 001.*

The quality of the cells was analyzed by flow cytometry and the expression of positive (CD90, CD166, CD73, CD105) and negative (CD31, CD45, CD14, CD34) surface markers was investigated for both the NANT 001 System and manually expanded cells by both Work Groups. The AD-MSCs expanded manually or by NANT 001 were compliant to the guidelines established by the ISCT (5), with the only exception of CD34, whose expression resulted quite variable. However, it is widely reported in literature that a high CD34 expression variability on AD-MSCs at early passages can be observed (7). Furthermore, cells grown in hPL can display higher levels of CD34 (8). Nevertheless, the levels of CD34 expressed by the cells cultivated in NANT 001 was on average lower than those obtained with the manual process (Figg. 8-10)

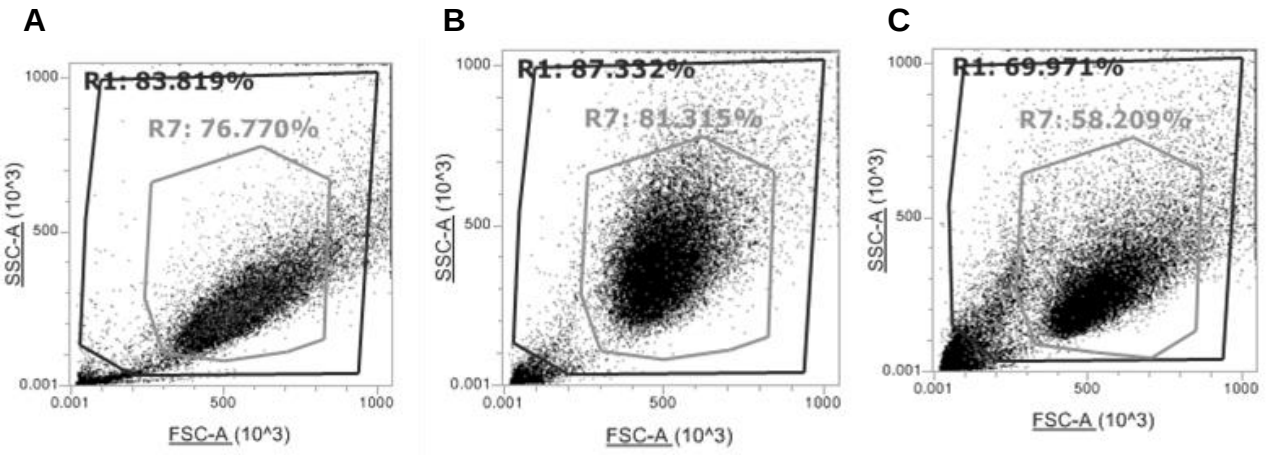

Figure 8. *Morphological analysis of harvested AD-MSCs performed by Work Group 1;* ***A****. Cells expanded in αMEM 4F medium and manual protocol;* ***B.*** *Cells expanded in αMEM 5% hPL medium and manual protocol;* ***C****. Cells expanded in αMEM 5% hPL medium in NANT 001 System.*

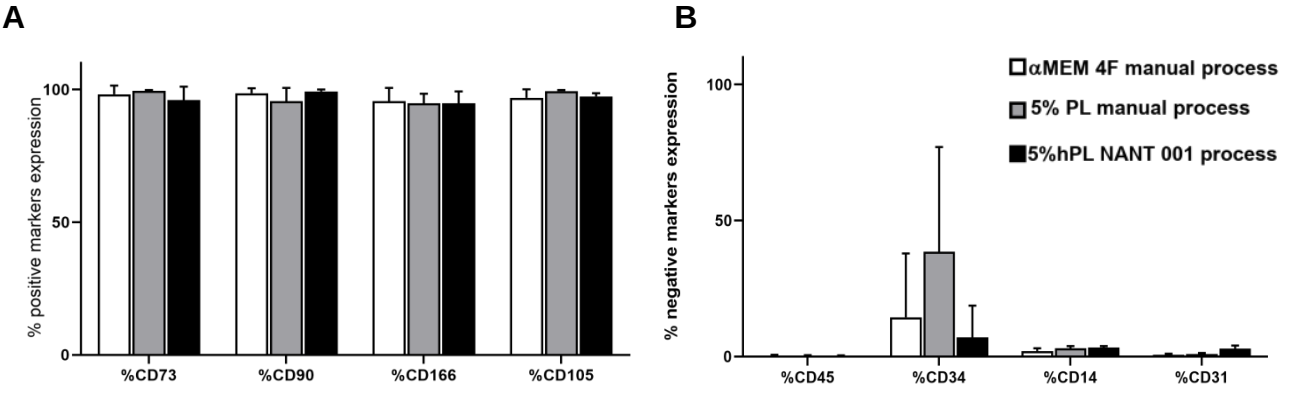

Figure 9. *Flow cytometry AD-MSCs surface markers analysis performed by Work Group 1;* ***A.*** *Positive markers analysis;* ***B.*** *Negative markers analysis.*

Work Group 2 performed the same flow cytometry analysis as Work Group 1 for the positive and negative surface markers, and all of the results were in line with the ISCT (5) guidelines except for CD34, as previously discussed for results of Work Group 1. Again, despite the high levels of CD34, the same cells expressed low levels of the negative marker CD45 and high levels of the positive markers CD166 and CD90 (Fig. 10), confirming that those cells may be actually classified as MSCs.

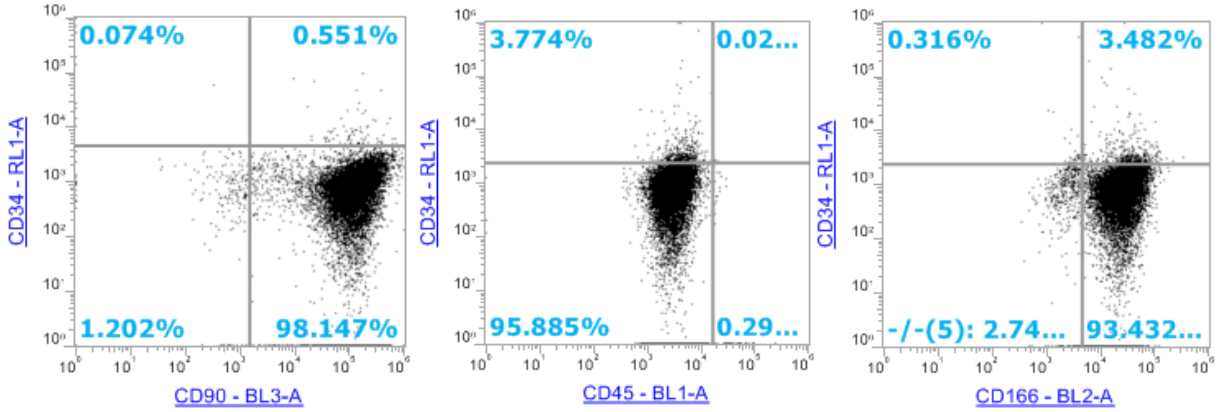

Figure 10. *Flow cytometry AD-MSCs surface markers analysis performed by Work Group 2. CD34 expression was analysed together with MSCs markers (CD90 and CD166) and hematopoietic stem cells marker (CD45), to verify that, despite the high levels of CD34, the harvested cells were actually MSCs.*

Interestingly, the variable percentage of CD34+/ CD90+/CD73+ cells observed during the analysis of the SVF by Work Group 1 (Table 3), seem to be correlated with a lower doubling time (Table 4). In literature, it is well described that the variable percentage of CD34, CD90, and CD73 is associated with the quantity of proliferating MSCs in the SVF (1-2-3) and with a higher number of cells that adhere to the plastic surface after 24h (4). Sample #4 was atypical, probably due to the small number of MNCs (see Table 2).

Table 4. *Summary of the correlation between cell surface markers in SVF and cell growth rate obtained by Work Group 1.*

	Days of culture	No. of harvested cells	Doubling Time (hours)	CD34+ CD90+ CD73+ (%) in SVF
Sample #1	8	53.5×10^6	39.9	14.3%
Sample #2	11	76.4×10^6	49.6	9.9%
Sample #3	12	41.4×10^6	64.8	7.2%
Sample #4	10	40.8×10^6	54.3	3.2%

CONCLUSIONS

The goal of this work was to show that the NANT 001 automated cell expansion System is able to efficiently support AD-MSCs expansion using a human Platelet Lysate-based (hPL), serum-free cell culture medium. Expansions were successfully performed in parallel by two different Work Groups, independently from the surface marker expression of the fresh starting material, confirming the reproducibility and robustness of the method.

The results obtained showed that the expansion process carried out with the NANT 001 System appears to be, for a certain extent, even more efficient than the manual process in terms of cell growth rate. In particular, cell density (cells/cm^2 of growth surface) obtained with the NANT 001 System using a serum free medium was higher not only with respect to that obtained in manual expansion with the same hPL-based medium, but also to the one obtained with a FBS supplemented medium.

Looking at the quality of the cells in terms of cell surface markers and viability, the results were comparable in all conditions, confirming that the NANT 001 System allows an efficient automated cell expansion, while maintaining the same quality as in manual expansion. All of the surface markers analyzed were between the range established by ISCT (5), with the only exception of CD34, whose expression was quite variable between the samples. However, it is widely reported in literature that there is high

variability on AD-MSCs at early passages and that cells grown in hPL showed higher levels of CD34 (6-7).

In this context, it is important to note that not only the expansion with the NANT 001 System is efficient, but also that the manipulation of the cells is minimized throughout the expansion process, preventing possible contamination. Indeed, the limitation of manual cell culture manipulation to isolation and downstream processes only, helps meeting the GMP manufacturing requirements in the field of Advanced Therapy Medicinal Products manufacturing (ATMPs) (9). Furthermore, all the in-process control parameters are constantly under control, and are conveyed in a Cell Culture Report summarizing all the events and parameters monitored during the expansion process, for a complete traceability of operations, that is a key aspect of Good Manufacturing Practices.

All these results confirm the ability of the NANT 001 System in conducting automated AD-MSCs expansions in a safe and reproducible way, and that the automated process performed with this System meets all the GMP requirements in terms of safety, traceability and reproducibility.

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