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CELL EXPANSION SYSTEM



Automated Expansion of Human Mesenchymal Stem Cells from
Precultured Cells Using the Quantum Cell Expansion System

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Abstract

The large number of ex vivo expanded cells that are required in many clinical cell therapy protocols (>200 million per patient) make standard culture conditions problematic and expensive, resulting in the need for extensive personnel and facilities resources, and the potential for contamination. To meet such clinical demand, a robust automated and closed cell expansion method is optimal. The Quantum system is a functionally closed, automated hollow fiber bioreactor system designed to reproducibly grow both adherent and suspension cells in either GMP or research laboratory environments. The system has been used for the ex vivo expansion of clinical-scale quantities of human mesenchymal stem cells (MSC). MSCs from precultured cells were expanded in the system with media consisting of α -MEM, 10 percent FBS, 1x GlutaMAX and no additional antibiotics/antimycotics or supplementary factors. Glucose and lactate levels in the media were monitored to maintain optimal culture conditions. Quantum system-expanded MSCs met all typical MSC characteristics for phenotype and differentiation. Cell numbers suitable for therapeutic dosages of MSC were generated in five days from initial cell loads of 15 to 20 million cells.

Quantum System

The Quantum system is a functionally closed, automated hollow fiber bioreactor system designed to grow both adherent and suspension cells (Figure 1). The bioreactor culture system is comprised of a synthetic hollow fiber bioreactor that is part of a sterile closed-loop circuit for media and gas exchange (Figures 1 and 2). The bioreactor and fluid circuit are a single-use disposable set that is mounted onto the Quantum system unit. The bioreactor itself is comprised of ~11,500 hollow fibers with a total intracapillary (IC) surface area of 2.1 m². Typical culture manipulations (e.g., cell seeding, media exchanges, trypsinization, cell harvest, etc.) are managed by the computer-controlled system using pumps and automated valves, which direct fluid through the disposable set and exchanges gas with the media. The functionally closed nature of the disposable set is maintained through the sterile docking of bags used for all fluids; these bag connections/disconnections all utilize sterile connection technology. Gas control in the system is managed using a hollow fiber oxygenator (gas transfer module, Figure 2). Gas is supplied from a user-provided premixed gas tank. By choosing a tank with the desired gas mixture, the user can expand cells at their optimal gas concentration. The IC membrane of the bioreactor is coated with an adhesion promoter to allow the attachment of adherent cell populations.



Figure 1. The Quantum system. The left image shows the Quantum system exterior with the door closed, in a working setting with media and waste bags attached. The right image shows the Quantum system's door opened, with the disposable set loaded.

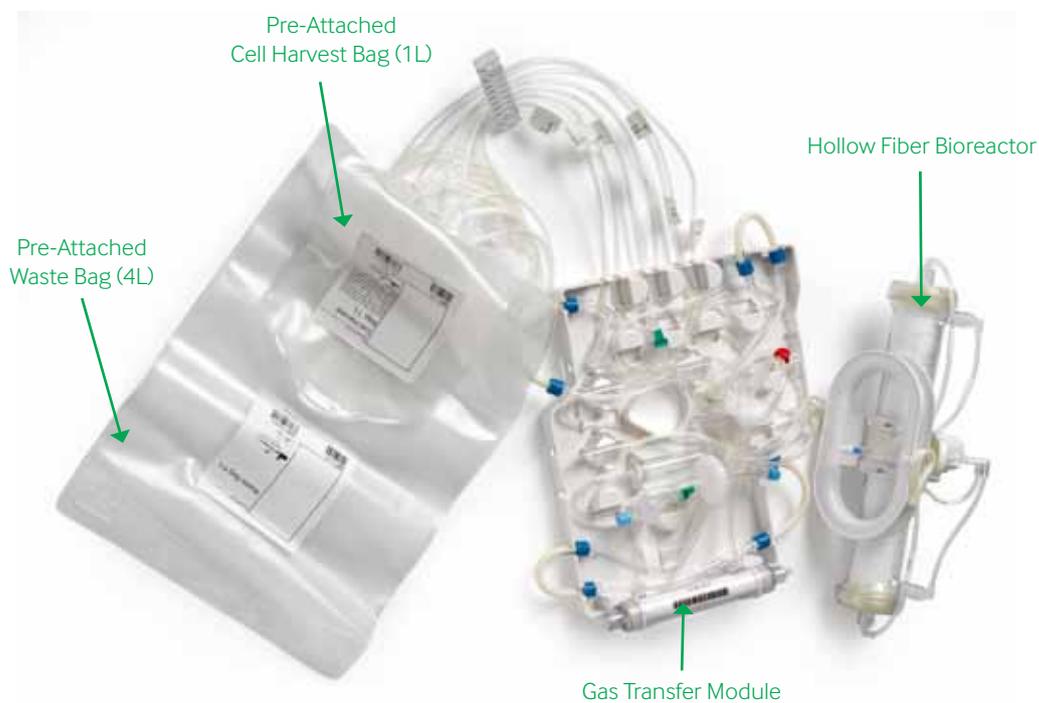


Figure 2. The Quantum system disposable set.

System Hydraulics

The Quantum system fluid circuit is designed around two fluid loops: one loop for IC and one for the extracapillary (EC) side of the bioreactor (Figure 3). Five different lines allow the connection of fluid bags to the Quantum system for cell loading, cell media support and feeding, waste removal and reagent addition. These are named the Cell Inlet Line, the Reagent Line, the IC Media Line, the Wash Line and the EC Media Line. As a practical matter, the content of the five bags is completely at the discretion of the user. The user may choose to add fluid from any selected bag(s) to the system at a flow rate and volume that can be chosen by the user. The flexibility of the system allows the choice of the addition of fluid to the IC loop simultaneously with the EC loop, or independently. The bioreactor membrane between the IC and EC spaces allows free gas diffusion between the IC and EC sides of the bioreactor, as well as small molecule diffusion so that glucose and lactate freely pass from one side of the membrane to the other. Larger macromolecules are sequestered on the side of the membrane in which they are added; for this reason it is important to make sure that media with large molecules critical for cell culture (e.g., Cytokines and growth factors) are loaded on the IC side when cells are expanded on the IC side of the bioreactor. The volume of the IC fluid circuit is 189 mL, and the volume of the EC fluid circuit is 305 mL. Fluid may exit the system to either the waste bag or the harvest bag. Because the system has a constant volume, all addition of fluid will result in an equal volume of fluid leaving the system. With the exception of cell harvest, the system always operates with an open fluid path to the waste bag.

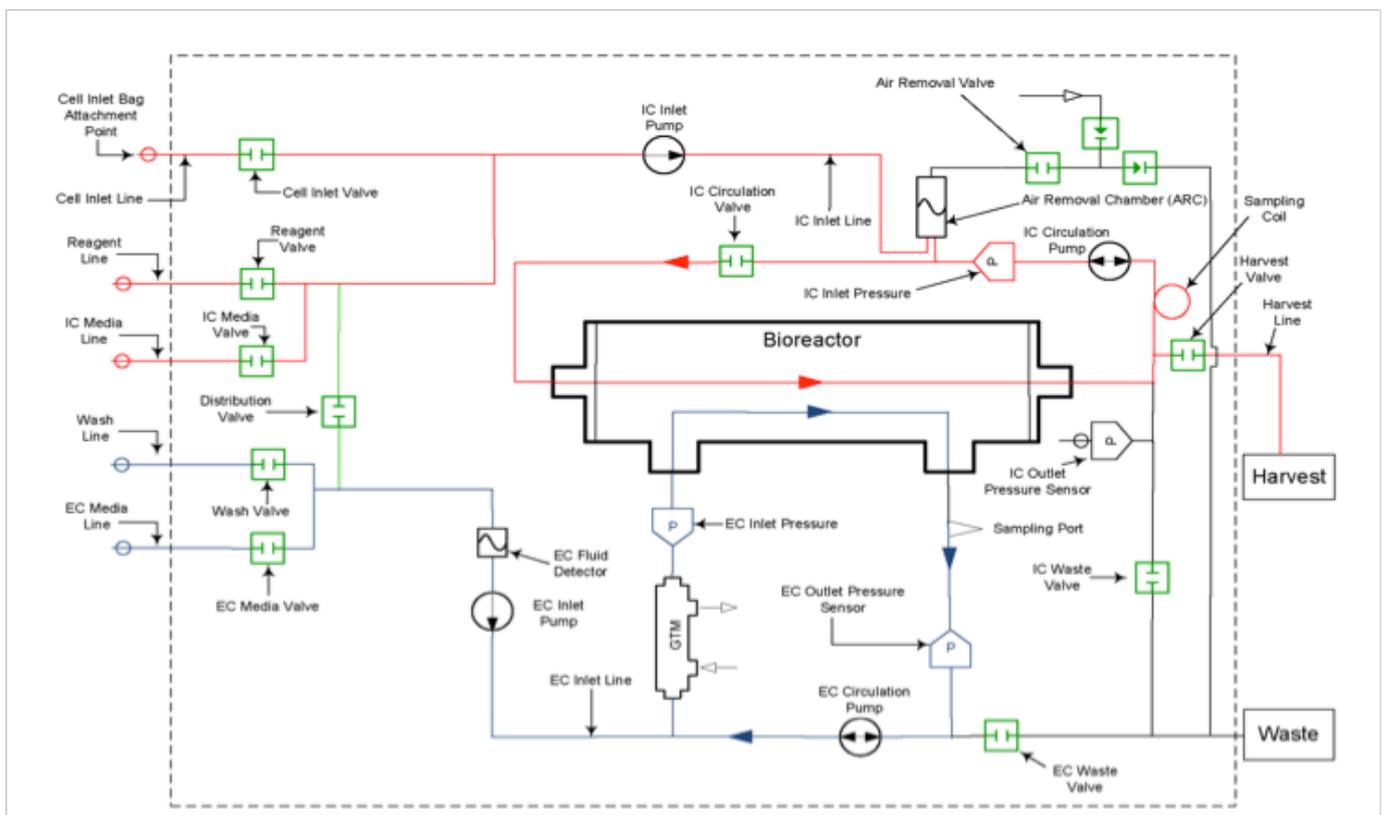


Figure 3. The Quantum system hydraulic layout with the IC loop shown in red, and the EC loop in blue.

Tasks

The Quantum system can perform operations ranging from a manual mode (Custom Task) to full automation of a specific process (Automated Task). The Quantum system's touch screen is used to select and perform these tasks. The touch screen not only contains buttons to enter data or to command the system to perform a specific action, but also displays real-time information about the condition of the system. The different functions operate the system's pumps and valves to perform the specified task. In all cases the user may accept the default values or may use the touch screen to input different parameters. The Automated Tasks allow the user to select and begin a task without entering any task settings manually, if the choice is to use the factory default settings for the selected task. The task categories are: Set Management, System Management, Washout, Load and Attach, Feed and Add, Release and Harvest, and Custom. Automated Tasks still offer flexibility because the user has the option to enter the task settings manually for an Automated Task. The values for each of the steps for a task may also be input by the user for each of the steps before beginning the task, which creates a seamless transition from step to step during the task. It is important to note that valves are managed only by the Quantum system's software, and it will allow the choice of only certain inlet and/or outlet lines according to the task that is going to be performed: for example, the software allows the Cell Inlet line to be a source of liquid only for the IC side during Cell Loading Tasks. For Custom Tasks the user must manually enter all the task settings and there are no default task settings. Custom Tasks can be saved by the user so that settings do not have to be entered again manually.



Figure 4. The Quantum system touch screen showing the Task Bar.

Expansion of Precultured MSC

The precultured MSC expansion protocol consists of culturing MSCs derived from human bone marrow using Complete Media in the system (10 percent FBS and 1x GlutaMAX in α -MEM) with no additional antibiotics/antimycotics or supplementary factors. In this study, 12 runs have been performed using Automated Tasks.

All work was performed at CaridianBCT Bio labs in Lakewood, Colorado.

The following materials and reagents were used in this study:

- Thawed liquid nitrogen-frozen precultured human bone marrow-derived MSCs
- Disposable Set, CaridianBCT catalog # 21012
- Cell Inlet Bags, CaridianBCT catalog # 21020
- Media Bags, CaridianBCT catalog # 21021
- Waste Bags, CaridianBCT catalog # 21023
- Phosphate Buffered Saline (PBS), 1 L bottles, Lonza catalog # 17-516Q
- Alpha MEM, 500 mL bottles, Lonza catalog # 12-169F
- Fetal Bovine Serum (FBS), 500 mL bottles, Lonza catalog # 14-501F
- GlutaMAX, 100 mL bottles, Gibco catalog # 35050
- Human Fibronectin, 5 mg vial, BD Biosciences catalog # 356008
- Trypsin, 0.25 percent with EDTA, 100 mL bottles, Gibco catalog # 25200
- Harvest Media (α -MEM, Lonza, catalog # 12-169F; 20 percent FBS, Lonza, catalog # 14-501F; 1x GlutaMAX, Gibco, catalog # 35050; D-Glucose, Sigma, catalog # G5767; Calcium Chloride, Sigma, catalog # C3306; Magnesium Sulfate, Sigma, catalog # M1880)

Disposable Set Loading and Cell Expansion Set Up

After loading a new cell expansion set using the Load Cell Expansion Set Task (every run/passage requires the use of a new disposable set to assure process sterility), the disposable set is primed with PBS in the 4 L Media Bag that has been connected to the Cell Inlet line of the disposable set using a Terumo® TSCD or TSCDII sterile connection device. The Media Bag Accessory is filled with fluid from reagent bottles under a biosafety cabinet utilizing a tubing pump (Cole-Parmer Masterflex® L/S Tubing Pump with the Easy-Load II pump header) to pump the fluid into the Media Bag Accessory; all sealing of disposable tubing lines is done with an RF Sealer (Sebra Omni™ 2600 Sealer). Once the disposable set has been primed, the bioreactor is coated overnight with 10 mg of fibronectin to promote cell adhesion using the Coat Bioreactor Task. The fibronectin is prepared by solubilizing 10 mg of fibronectin in 20 mL of sterile H₂O for 30 minutes, bringing the solution volume to 100 mL with 80 mL of PBS, transferring the fibronectin solution to a Cell Inlet Bag in a biosafety cabinet. After the overnight bioreactor coating, any excess fibronectin is washed from the bioreactor set and the cell culture media is introduced into the set utilizing the IC/EC Washout Task allowing the exchange of PBS solution with Complete Media, which has been filled into a 4 L Media Bag accessory and sterile connected to the IC Inlet Line. To assure that the newly introduced Complete Media is properly oxygenated, the Condition Media Task is run for a minimum of 10 minutes. The gas mixture used was 20 percent O₂, 5 percent CO₂ and the balance N₂. At this point the disposable set is ready to be used for MSC loading and expansion.

Precultured MSC Cell Loading and Expansion

Fifteen to twenty million thawed and washed precultured human bone marrow-derived MSC (cells were frozen and cryopreserved in liquid N₂ at Passage 2-4) are transferred into the Cell Inlet Bag and the total volume of the bag is brought up to 100 mL with Complete Media. The bag is then sterile-connected to Cell Inlet Line of the Quantum system and cells loaded onto the IC side of the bioreactor utilizing the Load Cells with Circulation Task. This task is designed to allow a uniform distribution of the cells throughout the IC side of the bioreactor. Once this task is completed, the system is put into the Attach Cells Task mode, which allows the cells to adhere to the IC membrane surface. For this task there is an IC media flow rate of zero to allow cell attachment, while the EC flow rate is 30 mL/min to maintain oxygenation in the system. Precultured MSCs are allowed to attach for 24 hours followed by a Rapid IC Washout to remove any nonadherent cells.

Cells are grown for four days utilizing the Feed Cells Task with the fresh Complete Media added to the IC side of the bioreactor and the IC inlet rate adjusted as required by the rate of glucose consumption and lactate generation in the system as sampled from the Sample Port twice daily (measured using a Siemens blood gas analyzer). If glucose values are below 75 mg/dL in the morning or 85 mg/dL in the afternoon, the rate is increased two-fold. Depending on the adjusted feed rates, a standard five-day MSC expansion typically consumes approximately 7.7 L of Complete Media when loading approximately 15 to 20 million MSCs. Typical IC inlet rates are 0.1 mL/min on Day 1, 0.2 mL/min on Day 2, 0.4 mL/min on Day 3, 0.8 mL/min on Day 4, and 1.6 mL/min on Day 5. The IC Waste Valve is open for the duration of the expansion phase to allow Waste Media to collect into the Waste Bag and to prevent protein accumulation in the IC loop.

Cell Release and Harvest

On Day 5, expanded MSCs are released from the IC membrane of the bioreactor using 0.25 percent trypsin-EDTA as the enzymatic release agent. A Cell Inlet Bag is filled with 180 mL of 0.25 percent trypsin-EDTA solution in a biosafety cabinet, and the bag is sterile-connected to the Reagent Line of the disposable set. The Release Adherent Cells Task is used to flush Complete Media from the system with PBS, then to fill the bioreactor with the trypsin solution that is circulated in the bioreactor for four minutes. Once this task is complete, released cells are harvested utilizing the Harvest Task with at least 0.6 L of Harvest Media in a Media Bag attached to the IC Inlet Line. Once the Harvest Task is complete, the expanded MSCs are located in the Harvest Bag containing approximately 438 mL of cells in Harvest Media. This Bag is sealed with an RF sealer and detached from the disposable set; the cells are removed from the bag for cell counting, viability, phenotype, morphology and differentiation assays.

| Day | Task | Media |
|-------|-----------------------------|--|
| -1 | Load Cell Expansion Set | None |
| | Prime Cell Expansion Set | PBS |
| | Coat Bioreactor (12 hours) | Fibronectin in PBS |
| 0 | IC/EC Washout | IC Inlet: Complete Media EC Inlet: Complete Media |
| | Condition Media | IC Inlet: None EC Inlet: Complete Media |
| | Load Cells with Circulation | IC Inlet: Complete Media EC Inlet: None |
| | Attach Cells (24 hours) | IC Inlet: None EC Inlet: Complete Media |
| 1 | Rapid IC Washout | IC Inlet: Complete Media EC Inlet: Complete Media |
| 1 - 5 | Feed Cells | IC Inlet: Complete Media EC Inlet: None |
| 5 | Release Adherent Cells | IC/EC Washout with PBS then Trypsin Load and Circulation |
| | Harvest Cells | IC Inlet: Harvest Media EC Inlet: Harvest Media |

Table 1. The schedule of tasks performed for each day during the five-day precultured MSC expansion process.

Precultured MSC Expansion Results

A total of 12 expansion runs yielded cell harvests averaging 336 million MSCs per bioreactor, with an average viability of 94 percent by 7AAD flow cytometry. Quantum system-expanded MSCs met typical MSC characteristics according to the ISCT position paper (Dominici M, et al., Cytotherapy 2006, 8: 315-317.) in terms of morphology (maintaining plastic adherence and spindle shape characteristics); differentiation (osteoblasts, adipocytes, and chondrocytes), and phenotype (positive for CD105, CD73 and CD90; and negative for CD45, CD34 and HLA class II). The harvest results of 12 precultured MSC expansion runs are summarized in Table 2.

| Run | Days Grown | Cells Harvested | Viability% (7AAD) | dT (hrs) | Number of Doublings |
|----------------------|------------|-----------------|-------------------|----------|---------------------|
| 1 | 5 | 2.81E+08 | 87.2 | 31.5 | 3.8 |
| 2 | 5 | 2.75E+08 | 89.2 | 31.7 | 3.8 |
| 3 | 5 | 2.99E+08 | 97.2 | 30.8 | 3.9 |
| 4 | 5 | 3.09E+08 | 95.4 | 30.4 | 3.9 |
| 5 | 5 | 3.18E+08 | 94.1 | 30.1 | 4.0 |
| 6 | 4.7 | 3.77E+08 | 93.3 | 26.6 | 4.2 |
| 7 | 4.7 | 3.56E+08 | 92.6 | 27.2 | 4.2 |
| 8 | 4.7 | 3.80E+08 | 91.7 | 26.6 | 4.2 |
| 9 | 4.7 | 3.57E+08 | 96.4 | 27.1 | 4.2 |
| 10 | 4.7 | 3.69E+08 | 94.3 | 26.8 | 4.2 |
| 11 | 4.7 | 3.30E+08 | 94.6 | 27.9 | 4.0 |
| 12 | 4.7 | 3.86E+08 | 96.5 | 26.4 | 4.3 |
| Calculations: | | | | | |
| Average | | 3.36E+08 | 93.5 | 28.6 | 4.1 |
| Standard Deviation | | 3.96E+07 | 3.0 | 2.1 | 0.2 |

Table 2. Summary of results for 12 cell expansion runs of precultured MSCs in the Quantum system.

Morphological characterizations of the cells post-harvest were similar among all the runs. Morphological characterization is performed by microscopic examination to determine whether the MSCs display plastic adherence within one day of harvest from the Quantum system, show growth within four days and have MSC spindle-shaped characteristics. All runs showed plastic adherence by Day 1 and growth by Day 4. See Figure 5 for observations.

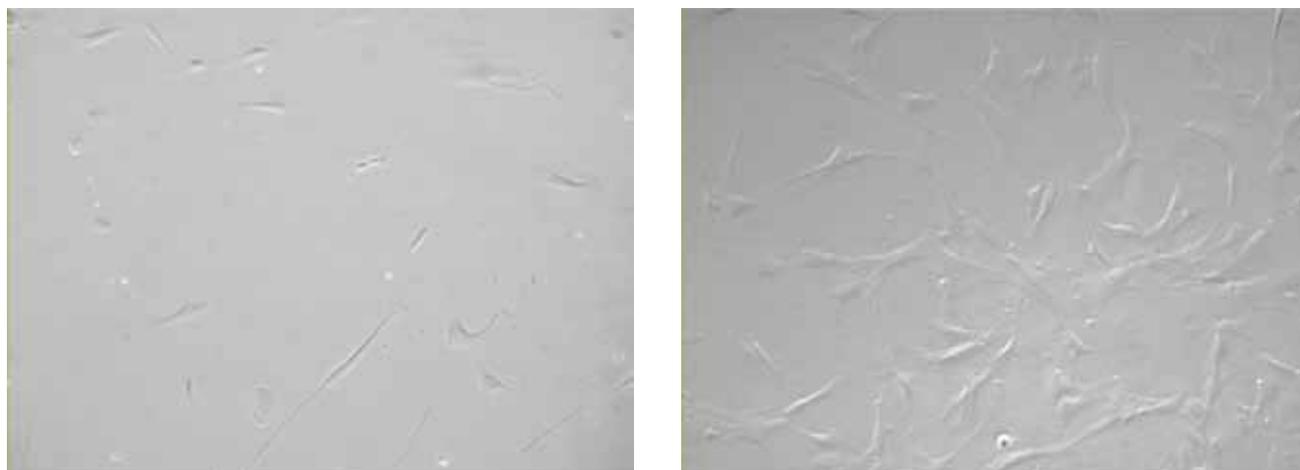


Figure 5. Typical MSC morphology on Day 1 (left image) and Day 4 (right image).

MSC differentiation into osteoblasts, adipocytes and chondroblasts was demonstrated for all 12 precultured MSC expansion runs. These assays use Oil Red O to stain for adipocytes, Alizarin Red to stain for osteocytes and Alcian Blue to stain for chondrocytes. A representative set of micrographs (400x) is shown in Figure 6.

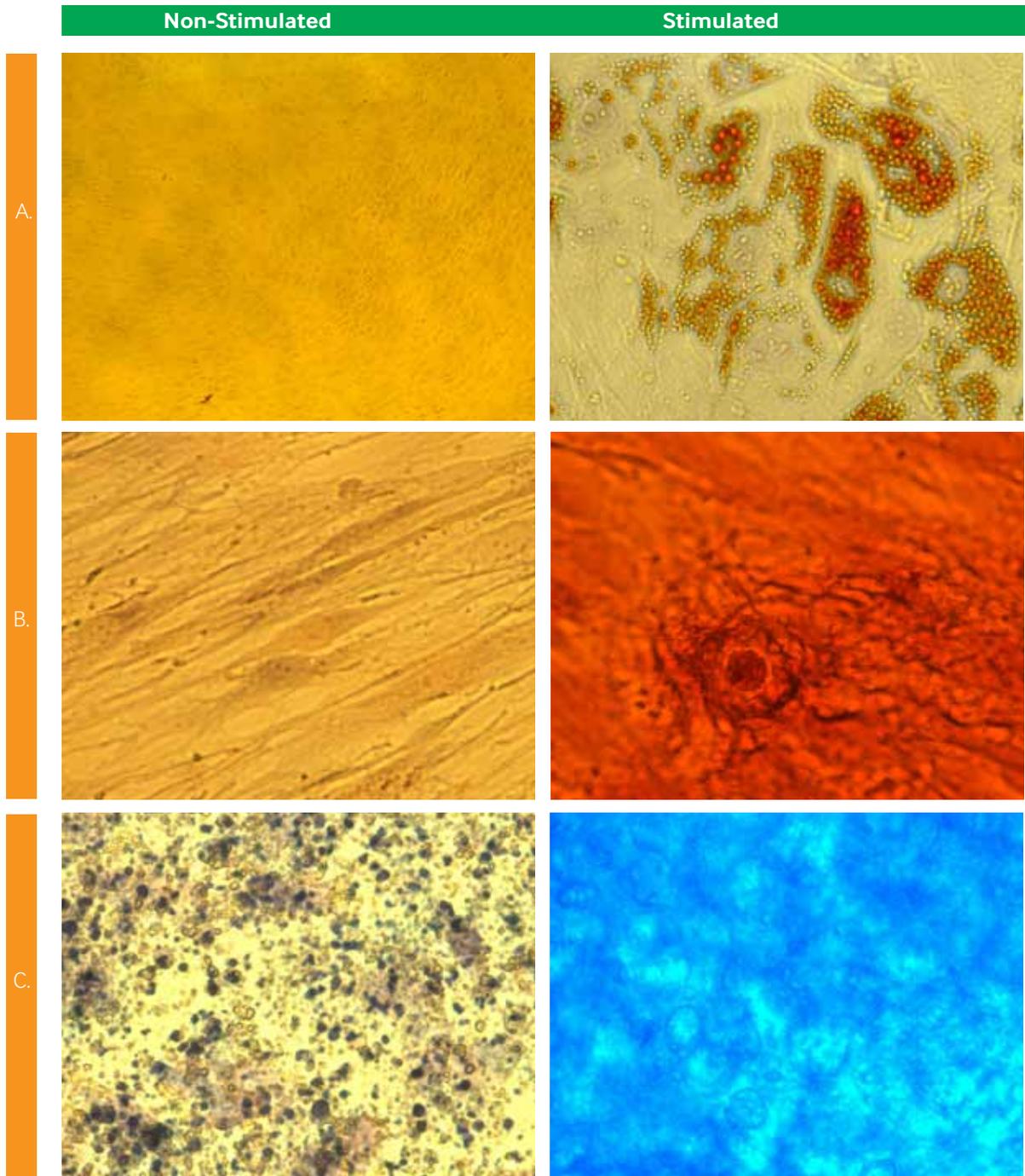


Figure 6. A. Oil Red O staining for adipocytes, B. Alizarin Red staining for osteocytes, and C. Alcian Blue staining for chondrocytes.

The flow cytometry results were similar among all the runs and the percent positives of each biomarker were within the acceptance ranges for MSCs as defined in the ISCT position paper (refer to Table 3). The biomarkers CD34, CD45, and HLA-DR should not be expressed in MSC and have low percent positives. All the runs were below 2 percent positive for these biomarkers. The biomarkers CD73, CD90 and CD105 should be expressed in MSC and have high percent positives because they are a requirement for MSC. All the runs were 99 percent positive or more for these biomarkers.

| Run | CD34 (%+) | CD45 (%+) | CD73 (%+) | CD90 (%+) | CD105 (%+) | HLA-DR (%+) | 7AAD (%+) | |
|----------------------|-----------|-----------|-----------|-----------|------------|-------------|-----------|--|
| 1 | 0.34 | 1.28 | 98.47 | 96.11 | 98.41 | 0.64 | 12.85 | |
| 2 | 0.32 | 0.58 | 98.82 | 96.76 | 98.43 | 0.17 | 10.81 | |
| 3 | 0.06 | 0.22 | 99.54 | 97.85 | 99.66 | 0.01 | 2.83 | |
| 4 | 0.08 | 0.38 | 99.52 | 98.48 | 99.51 | 0.03 | 4.57 | |
| 5 | 0.04 | 0.19 | 99.46 | 98.39 | 99.39 | 0.05 | 5.89 | |
| 6 | 0.01 | 0.24 | 99.46 | 98.93 | 99.35 | 0.01 | 6.72 | |
| 7 | 0.05 | 0.26 | 99.60 | 97.67 | 98.80 | 0.05 | 7.45 | |
| 8 | 0.06 | 0.20 | 99.45 | 97.88 | 99.41 | 0.18 | 8.26 | |
| 9 | 0.09 | 0.12 | 99.72 | 98.22 | 99.52 | 0.01 | 3.59 | |
| 10 | 0.02 | 0.06 | 99.76 | 98.58 | 99.51 | 0.01 | 5.66 | |
| 11 | 0.03 | 0.05 | 99.54 | 98.69 | 99.63 | 0.04 | 5.45 | |
| 12 | 0.07 | 0.09 | 99.48 | 98.57 | 99.68 | 0.02 | 3.47 | |
| Calculations: | | | | | | | | |
| Average | 0.10 | 0.31 | 99.40 | 98.01 | 99.28 | 0.10 | 6.46 | |

Table 3. Flow cytometry of MSC markers for 12 precultured MSC expansion runs on the Quantum system.

Conclusion

The Quantum system has been used for automated ex vivo expansion of clinical-scale quantities of human MSCs. MSCs from precultured cells were expanded on the Quantum system with medium consisting of α -MEM, 10 percent FBS, 1x GlutaMAX and no additional antibiotics/antimycotics or supplementary factors. Cell numbers suitable for therapeutic dosages of MSCs were generated in five days from initial cell loads of 15 to 20 million cells. Cells were harvested after a maximum of five days in culture. A total of 12 expansion runs yielded cell harvests averaging 336 million MSCs per bioreactor, with an average viability of 94 percent by 7AAD flow cytometry. Quantum system-expanded MSCs met typical MSC characteristics according to the ISCT position paper (Dominici M, 2006) in terms of morphology, differentiation and phenotype.

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