Application Note

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Real-Time Quality Control and Functional Assessment of Mesenchymal Stem Cells for Cellular Therapies

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Abstract

A label-free assay for the functional assessment of bone marrow-derived mesenchymal stem cells (BMSCs) was developed using the Agilent xCELLigence real-time cell analysis (RTCA) instrument. This noninvasive method allows for continuous sampling of cellular status under physiologically relevant conditions. Multiple quantitative parameters can be derived from the data to characterize BMSCs and estimates cell potency and function.[1] This set of kinetic parameters is able to effectively estimate BMSC functional capacity (that is, differentiation potential), and can serve as a tool for preclinical quality control assessment.

Introduction

Mesenchymal stem cells (MSCs) are of great interest in regenerative medicine because:·

- They can differentiate *ex vivo* or *in vivo* into adipocytes, chondrocytes, and osteocytes, which can be used for tissue repair[2]
- They can suppress immune rejections and inflammatory responses when used alone or with other treatments

To generate MSCs for preclinical studies, cells from donors must be expanded over several passages *in vitro*. Because successive passages could reduce proliferation rates and cause loss of differentiation potential and high variability in functional capacity from one donor to the next, assessing the function of cells during and after the expansion process is critical.[3]

Currently, there are over 650 clinical trials worldwide using bone marrow-derived MSCs (BMSCs), with phase II trials spanning osteoarthritis and spinal cord injury to myocardial infarction and neurological disease.[4] However, advancements in this field have been impaired by a lack of cell-to-cell consistency and quality control (QC) standards, leading to failed or misinterpreted results. Studies suggest that the minimum criteria for defining MSCs, set by the International Society for Cellular Therapy (ISCT) in 2006, are not predictive of stem cell potency[5,6] (Table 1). Current assays lack the ability to quantitatively monitor cellular yields in real time, and simultaneously provide functional characterization and assessment of the product's quality parameters, such as viability, purity, and potency.[7]

Table 1. Minimum criteria for characterizing MSCs, as defined by the ISCT.[6]

Characteristic	Parameter
Adherance	Adherence to plastic tissue culture plate/flask during isolation step to distinguish from nonadherent contaminating cells
Positive markers: CD73, CD90, CD105	≥95% measured by flow cytometry
Negative markers: CD45, CD34, CD14 or CD11b CD79alpha or CD19, HLA-DR	≤2% measured by flow cytometry
Differentiation potential	Defined by staining of differentiated osteoblasts, adipocytes, and chondroblasts

Using cellular impedance to analyze MSC proliferation

The functional unit of the Agilent xCELLigence RTCA impedance assay is a set of gold biosensors embedded within the bottom surface of a microplate well (Agilent E-Plate, Figure 1). When submersed in media, applying a weak electric potential across these electrodes causes an electric current to flow between them. Because this phenomenon is dependent upon the electrodes interacting with bulk solution, the presence of adherent cells at the electrode-solution interface impedes the flow of the current. The magnitude of this impedance depends on the number of cells, the size of the cells, cell-substrate attachment quality, and cell-cell adhesion (barrier function). When BMSCs from

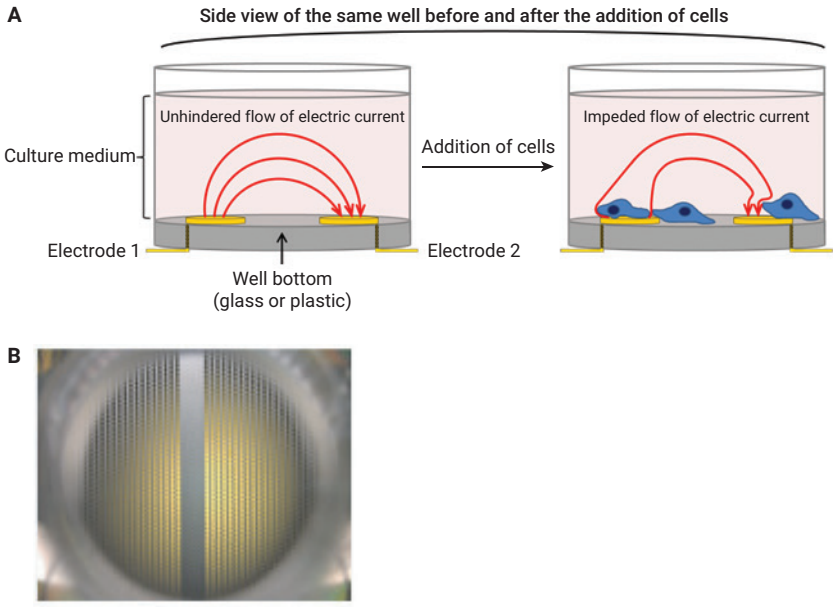


Figure 1. Overview of cellular impedance apparatus. (A) A side view of a single well from an Agilent xCELLigence electronic microplate (E-Plate). In the absence of cells, electric current flows freely through culture medium, completing the circuit between the electrodes. As cells adhere and proliferate on the biosensors, current flow is impeded, providing a sensitive readout of cell number, cell size, cell-substrate attachment quality, and cell-cell adhesion (barrier function). Note: not drawn to scale. (B) Photograph looking down into a single well of an E-Plate. In contrast to the simplified scheme in part A, the electrodes are actually an interdigitated array that covers >70% of the well bottom. Though cells can also be seen on the gold biosensor surfaces, the biosensor-free region in the middle of the well facilitates microscopic imaging.

different donors, different passage numbers, or from different culture conditions are seeded on E-Plates, differences in cellular status and properties are captured and quantified through real-time impedance signals, thereby providing rigorous and highly reproducible cellular characterization.

Protocol: Using xCELLigence RTCA to study MSCs

Agilent xCELLigence instrument

All impedance experiments in this study were conducted using Agilent 96-well electronic microplates (E-Plate 96) on an xCELLigence real-time cell analysis (RTCA) multiple plates (MP) instrument that was housed in a standard tissue culture incubator, set to maintain 37 °C and a 5% CO_2 atmosphere. All tests were performed in biological replicates of six. Impedance values recorded by the xCELLigence instrument were converted to a parameter Cell Index (CI), which is defined as $(Z_n - Z_b)/15$, where Z_n and Z_b are the impedance values in the presence and absence of cells, respectively.

Cells, media, and assay details

For experiments comparing different growth conditions, cells from the same donor were grown in MSC basal medium containing 7% FBS (serum) for two passages to condition the cells following thawing. Beginning at passage four (P4), cells were successively passaged in serum-containing media, serum-free media (SFM), or SFM Xeno-free (XFM) in T75 flasks. For cells grown in SFM or XFM, all culture plates and flasks were coated with Cellstart Substrate (Thermo Fisher). Every two passages, a subset of cells were seeded in E-Plates where attachment and growth were monitored using the xCELLigence instrument for at least 10 days.

The following data acquisition schedule was used: one read every 30 seconds for eight hours, followed by one read every 15 minutes for 16 hours, followed by one read per hour for 400 hours. Media changes were done every two to three days.

For experiments assessing donor-to-donor variability, cells were bought from ATCC, Thermo Fisher and Lonza. The donors, referred to as donors 1-3, were chosen to reflect differences in age, gender, and race. Cells from all three donors were grown in XFM and propagated as above, with cryopreservation of an aliquot of cells at every passage. To characterize these samples, cells from multiple passages were thawed out simultaneously and allowed to recover in T75 flasks. When cells reached ~80% confluence, they were seeded in E-Plates and growth was monitored as above.

For experiments assessing differentiation potential, cells from select passages were induced for osteoblast, adipocyte, and chondroblast differentiation using StemPro differentiation kits (Thermo Fisher), followed by staining according to the manufacturer's instructions.

For phenotypic characterization of BMSCs, cells were washed and stained with a panel of antibodies (Figures 4 and 6, E and K) for 30 minutes in PBS+10% FBS. After washing in PBS+1%FBS, cells were resuspended in PBS+1%FBS, and analyzed using a NovoCyte flow cytometer.

Defining quantitative parameters for quality control

Cell Index doubling time

The Cell Index doubling time (CI_{DT}) is calculated by the RTCA software, from the logarithmic phase of the growth curve (Figure 2, Curves A, CI_{DT}). CI_{DT} is a readout of cell proliferation and behavior that integrates changes in cell number, attachment, and morphology. In the example in Figure 2, the CI_{DT} of cell B is larger (more hours) than that of cell A, suggesting that changes in cell behavior occur at a faster rate in cell A than cell B.

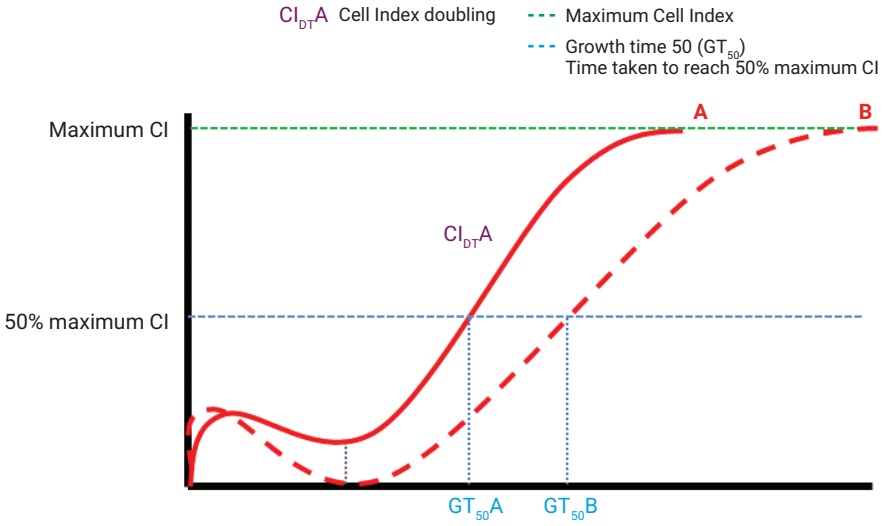


Figure 2. Overview of quantitative parameters derived from RTCA growth curves. Representative growth profiles comparing cell line A and cell line B. The graph represents how maximum CI (CI_{max}) and 50% maximum CI (GT_{50}) are calculated (blue lines). CI_{DT} represents the time it takes for the CI to double (solid red), and is calculated for the entire log growth phase.

Maximum cell index and relative maximum CI

The maximum CI (CI_{max}) represents the Cell Index where cells have reached full confluency and maximal attachment strength (Figure 2). To account for any changes that occur in CI_{max} after the first experimental passage, the maximum CI in this first passage was set to 1.0, and all successive passages are analyzed as a relative change, referred to as relative maximum CI (RCI_{max}).

Growth time 50

Growth time 50 (GT_{50}) is the time needed for the MSCs to reach 50% of the CI_{max}. GT_{50} may be used to compare proliferation rates of multiple cell types, or different seeding densities of the same cell type. For example, in Figure 2, both cells reach the same CI_{max}, but the GT_{50} of cell A is faster than that of cell B, indicating a higher proliferation rate. To account for any changes in GT_{50} after the first experimental passage, the GT_{50} can be graphed over multiple passages, and the slope can be calculated by other methods in addition to Excel to represent the change in GT_{50} over time (Figure 3C).

Table 2. Quantitative parameters for quality control.

Parameter	Definition	Threshold*
CI_{DT}	Cell Index doubling time	CIDT ≤50
RCI_{max}	Relative maximum Cell Index	RCI_{max} ≥0.8
GT_{50}	Time it takes to reach 50% of the CI_{max}	See slope
S	Slope of GT_{50} over time	S <6

* Threshold that correlates with positive differentiation potential.

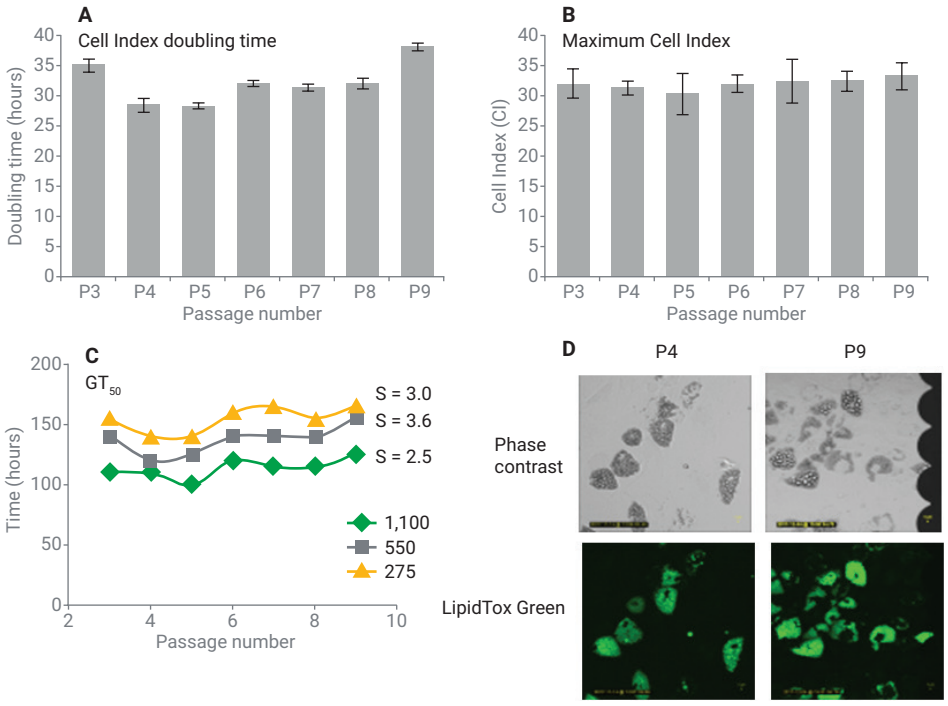


Figure 3. Quantitative parameters are reproducible and consistent over time for functional BMSCs. BMSCs were propagated over 12 passages in serum-containing media, and a subset of cell samples were seeded on Agilent E-plates on different experiment dates for each passage. Cells were induced to differentiate at passages 4, 6, and 9 to check for function. (A) CI doubling over different passages; (B) CI_{max} over different passages; (C) GT_{50} over different passages. Slope (S) was calculated for each seeding density (275, 550, or 1,100 cells/well), as a measure of change over time. (D) LipidTox Green lipid stain after 14 days of growth in adipogenesis differentiation medium (D).

Results and discussion

Quantitative xCELLigence parameters are consistent and reproducible over time

Based on recent results, BMSCs exhibit slower proliferation rates after multiple passages, and this correlates with loss of differentiation potential.[2] The exact passage number where this loss of function occurs varies significantly depending on cell donor and growth conditions, and currently there is no standard quality control assay to characterize this stage.

We sought to establish measurable quantitative parameters that could be compared to known standards to predict loss of function. BMSCs from donor 1 grown in serum-containing media were propagated over 14 passages in T75 culture flasks, with aliquots from each passage being saved in liquid nitrogen. Representative cells from different passages were thawed then seeded on E-Plates and monitored by RTCA. The results indicated that all three parameters CI_{DT}, (Figure 3A), CI_{max} (Figure 3B), and GT_{50} (Figure 3C), were consistent over successive passages through P9. Maximum variability from the mean was 5.2 and 10.5% for maximum CI and CI_{DT}, respectively. The slope of GT_{50} over time was nearly 0 (S = 2.5, 3.0, and 3.6 for each seeding density), suggests little to no variability in growth rate. The differentiation function of cells through P9 was tested for all three lineages. Based on their positive staining with the LipidTox Green lipid stain, P4 and P9 BMSCs can successfully differentiate into mature adipocytes (Figure 3D). P4 and P9 also showed a similar ability to differentiate into osteoblasts and chondroblasts (data not shown). The successive passages represent experiments carried out over the course of three months. As long as the cells still retained differentiation abilities, the quantitative parameters derived from RTCA testing were consistent, indicating the robustness and reproducibility of this RTCA assay.

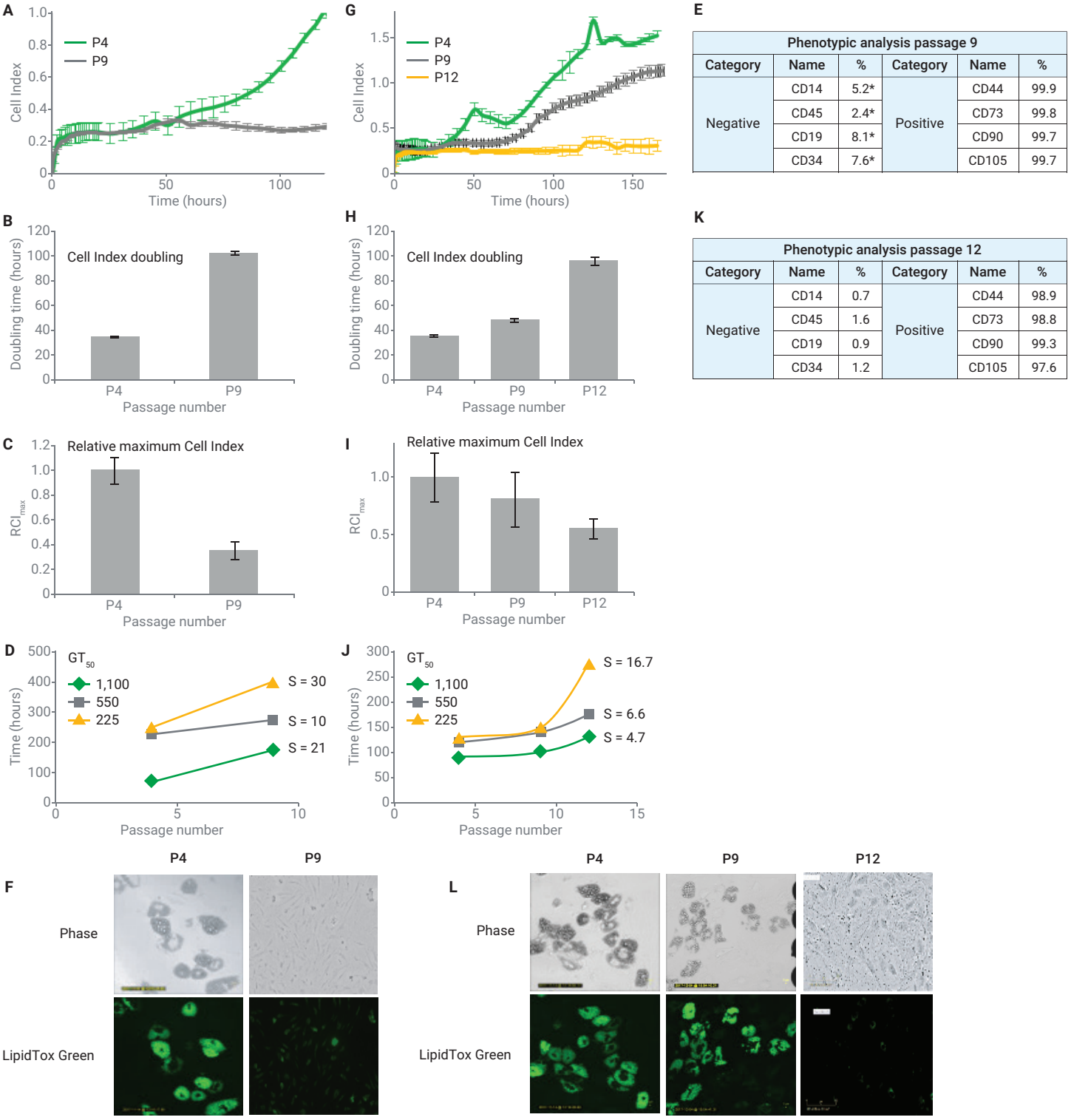
Quality control assessment of reagents for BMSCs culture

To evaluate BMSCs propagated under different culture conditions, the growth kinetics of cells expanded in serum-containing media, SFM, and XFM, were compared for early (P4), middle (P9), and late (P12) passages. Figure 4 contrasts the results for cells grown in SFM (Figure 4A-F) versus cells grown in XFM (Figure 4G-L). The kinetic growth profiles suggested that by P9 cells maintained in SFM had stopped proliferating (Figure 4A), while cells maintained in XFM continued to proliferate through P9 (Figure 4G).

Based on a combination of empirical observation and an exhaustive analysis of the quantitative xCELLigence-based parameters described above, each kinetic parameter (CI_{DT}, CI_{max}, and GT_{50}) was assigned thresholds used for estimating the differentiation potential of cells (Table 2). The data indicates that when CI_{DT} is greater than 50 hours, there is a loss of differentiation potential. This is seen at P9 for cells growing in SFM (Figures 4C and 4F) and at P12 for cells growing in XFM (Figures 4G and 4L). Similarly, when RCI_{max} is below 0.8, and S for GT_{50} is >6 for at least one seeding density, differentiation capacity of BMSCs is diminished (Figures 4C, 4D, 4E, 4I 4J, and 4L). Significantly, the predictivity of the xCELLigence-derived kinetic parameters/thresholds shown in Table 2 is confirmed by the phenotype staining LipidTox Green assay. Whereas cells maintained in SFM lost differentiation potential between P4 and P9 (Figure 4F), cells maintained in XFM retained differential potential through P9 (Figure 4L).

To determine the passage in which loss of function occurred, the user can calculate S for GT_{50} over different time intervals (passage numbers). Using the XFM-maintained cells as an example, the calculated S for the broad temporal window of P4 to P12 is 4.7, 6.6, and 30, for cell seeding densities of 1,100, 550, and 275 cells/well, respectively (Figure 4J). Breaking this slope analysis into finer temporal increments reveals exactly where loss of function occurred. The S for P4 to P9 is 2, 4, and 4 for the different cell seeding densities. Because S <6, it is clear that the cells are still functional at P9. In contrast, the S for P9 to P12 jumps to values of 10, 11.7, and 67 for the different cell seeding densities (data not shown). These kinetic parameters clearly indicate that the XFM cells lost function after P9, consistent with the findings of the LipidTox Green analysis.

BMSC surface marker phenotypic analysis was performed across multiple passages for each growth condition. The results supported previous findings that the minimum criteria set forth by ISCT for defining BMSCs are not predictive of function.[4,5] This is highlighted by the fact that at P12, BMSCs growing in XFM display a normal phenotype (Figure 4K), despite demonstrating complete loss of function (Figure 4L). We propose that by integrating the three quantitative parameters derived from xCELLigence testing, users can identify loss of function more accurately.



E

Phenotypic analysis passage 9					
Category	Name	%	Category	Name	%
Negative	CD14	5.2*	Positive	CD44	99.9
	CD45	2.4*		CD73	99.8
	CD19	8.1*		CD90	99.7
	CD34	7.6*		CD105	99.7

K

Phenotypic analysis passage 12					
Category	Name	%	Category	Name	%
Negative	CD14	0.7	Positive	CD44	98.9
	CD45	1.6		CD73	98.8
	CD19	0.9		CD90	99.3
	CD34	1.2		CD105	97.6

Figure 4. Quality control assessment of BMSCs under different culture conditions. BMSCs were propagated over 14 passages in serum-free media (A-F) or Xeno-free serum-free media (G-L), and a subset of cell samples from different passages were analyzed for quantitative parameters on an Agilent xCELLigence instrument. (A and G) Growth profiles comparing early and late passage under each growth condition. RTCA software was used to calculate Cell Index doubling (B and H) and relative CI_{max} (C and I) as described in the protocol. The dotted lines represent the threshold set for functional status. (D and J) GT_{50} was determined for successive passages. Slope (S) was calculated as a change over time for three different seeding densities (E and K). Phenotypic analysis of negative and positive MSC markers at the indicated passage where loss of function is observed. * Represents a value that is over the set threshold outlined in Table 1. (F and L) Differentiated staining at indicated passages with LipidTox Green lipid stain, following 14 days of growth in adipogenesis differentiation medium.

Quality control assessment of donor-to-donor variability

The same QC parameters and established thresholds described above (Table 2) were also used to assess donor-to-donor variability, and determine if the RTCA assay can estimate loss of function of BMSCs obtained from different sources. BMSCs obtained from different donors were propagated in XFM for at least 12 passages, and analyzed by RTCA. Representative results from the growth profiles for P2, P6, and P10 in Figure 5 suggested that there were significant qualitative differences in proliferation among the different donors.

While donor 3 exhibited continued proliferation into P10 (Figure 5C), proliferation rates for donor 2 and donor 1 slowed by P6 and P10, respectively (Figure 5A, 5B). Specifically for donor 2, CI_{DT}, was >50 (Figure 6B), RCI_{max} <0.8 (Figure 6C), and S for GT_{50} was >6 (Figure 6D) by P6, suggesting loss of function for donor 2 by this passage.

Conversely, for donor 3, all three parameters met the required thresholds (Table 2) by P6, but failed by P12 (Figures 6H, 6I, and 6J). This functional QC data from the xCELLigence assay was corroborated by the lack of adipocyte staining in P6 for donor 2 (Figure 6F). In contrast, for donor 3, we observed successful staining of mature adipocytes at P6, but no longer at P12 (Figure 6L).

Phenotypic stem cell surface marker analysis showed that, consistent with RTCA and differentiation results, BMSCs from donor 2 and donor 3 showed negative marker expression greater than the 2% limit at P6 and P12, respectively, for only three out of four markers (Figures 6E and 6K). However, little or no change was observed in the phenotypic expression of positive MSC markers at these specific passages. For donor 2, no changes were observed in expression of positive markers until P8 (data not shown), further reinforcing that these traditional guidelines may not be predictive of MSC potency because of the loss of function at P6.

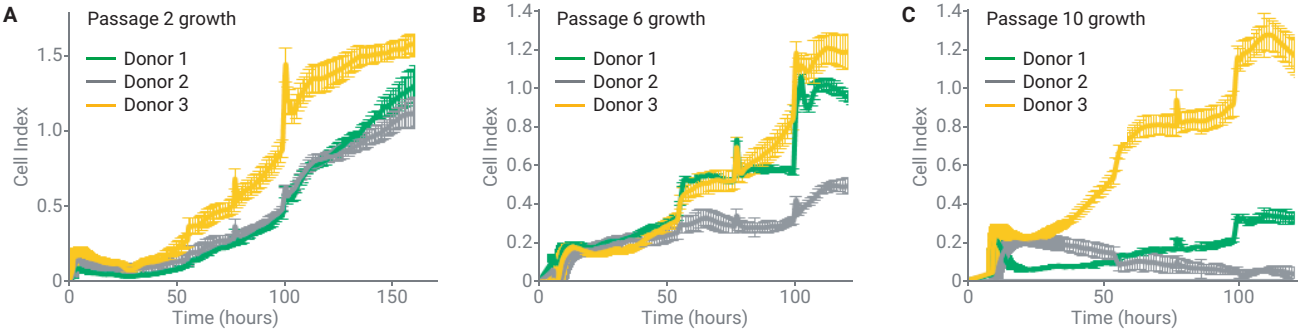
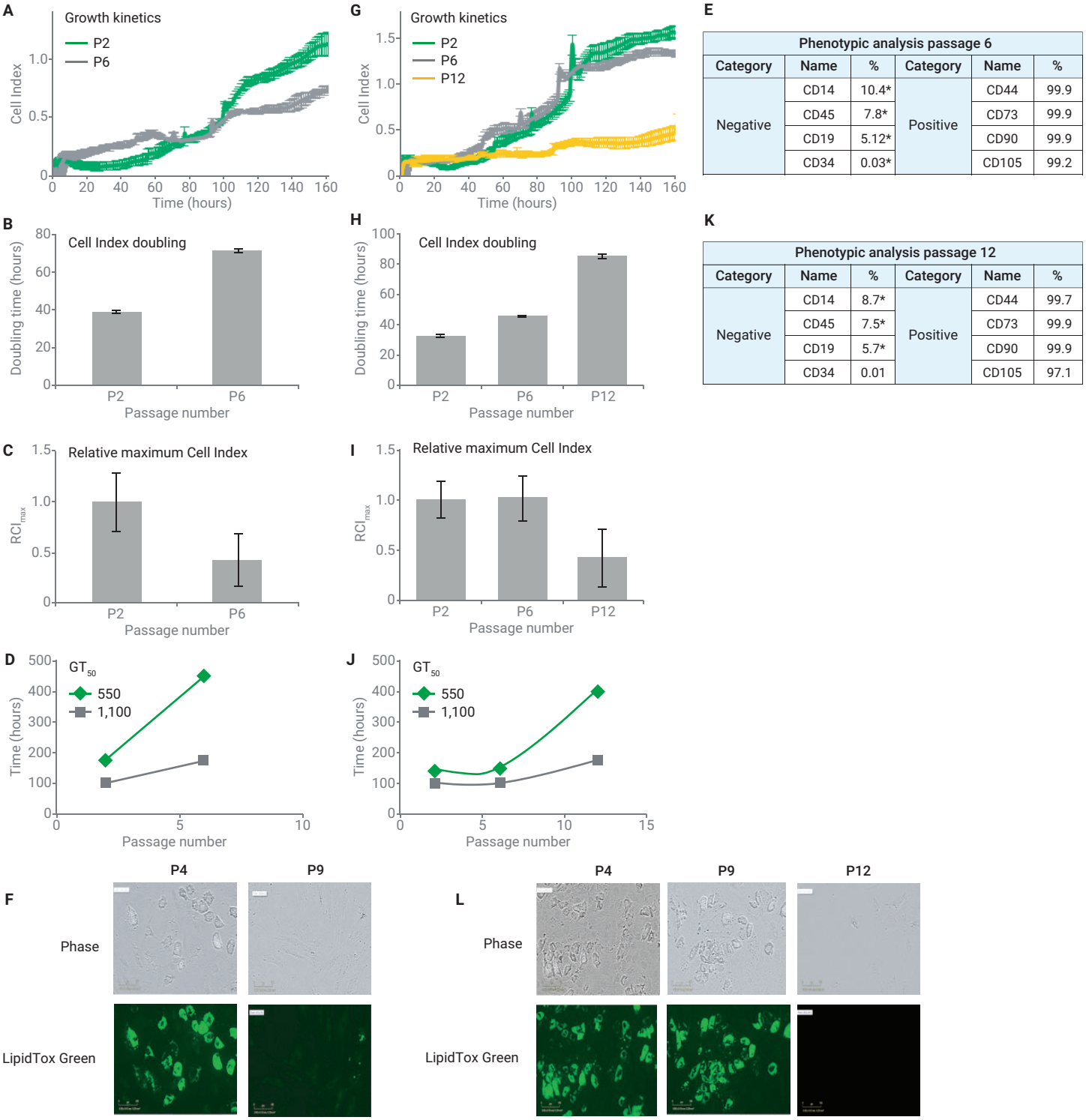


Figure 5. Real-time kinetic profiles of BMSCs from three different donors over multiple passages depict significant variation in growth potential. BMSCs from different donors were propagated over 12 passages in Xeno-free media, and a subset of cell samples from each passage were seeded on Agilent E-plates and monitored over 10 days. Representative growth profiles from passage 2 (A), passage 6 (B), and passage 10 (C) comparing all three donors.



E

Phenotypic analysis passage 6					
Category	**Name**	**%**	**Category**	**Name**	**%**
Negative	CD14	10.4*	Positive	CD44	99.9
	CD45	7.8*		CD73	99.9
	CD19	5.12*		CD90	99.9
	CD34	0.03*		CD105	99.2

K

Phenotypic analysis passage 12					
Category	**Name**	**%**	**Category**	**Name**	**%**
Negative	CD14	8.7*	Positive	CD44	99.7
	CD45	7.5*		CD73	99.9
	CD19	5.7*		CD90	99.9
	CD34	0.01		CD105	97.1

Figure 6. Quality control assessment of donor-to-donor variability in BMSCs. BMSCs from donor 2 (A-F) and donor 3 (G-L) from Figure 5 were propagated over 12 passages in Xeno-free serum-free media and a subset of cell samples from different passages were analyzed for quantitative parameters on an Agilent xCELLigence instrument. (A and G) Growth profiles comparing early and late passage under each growth condition. RTCA software was used to calculate Cell Index doubling (B and H) and relative CI_{max} (C and I) as described in the protocol. The dotted lines represent the threshold set for functional status. (D and J) GT_{50} was calculated for successive passages and plotted for two seeding densities. Slope (S) was calculated as a change over time for each seeding density. (E and K) Phenotypic analysis of negative and positive MSC markers at the indicated passage where loss of function is observed. * Represents a value that is over the set threshold outlined in Table 1. (F and L) Differentiated staining at indicated passages with LipidTox Green lipid stain, following 14 days of growth in adipogenesis differentiation medium.

Conclusion

Current QC assays in the field of cellular therapy, particularly for MSCs, lack reliable standards to rapidly and quantitatively characterize cells in real time without reducing manufacturing yields. High variability and lack of cell-to-cell consistency therefore continue to limit the utility of MSCs. xCELLigence RTCA can provide a functional assessment of MSCs. A small subset of cells is needed for the RTCA assay (helping to maximize production yields), and cells can be analyzed at any point during their propagation/differentiation or be cryopreserved and analyzed later. The biologically relevant parameters derived from the kinetic proliferation assay are consistent across multiple passages while cells continue to proliferate and differentiate into their respective lineages. Failure to meet the quantitative standards of this set of functional values indicates loss of differentiation potential, as observed in the donor-to-donor variability experiments (Figure 6). This xCELLigence-based QC assay can be applied to characterize variability resulting from the growth media, passage number, and batch consistency.

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