TheWell BIOSCIENCE

3D Cell Culture and Beyond

RocketCell® hMSC Xeno-Free Complete Growth Medium

High-performance, chemically-defined kit for superior expansion of human mesenchymal stem cells (hMSC)

Protocol

Catalog #: RC06-GM

Revision No: v1.0
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Human Mesenchymal Stem Cell (hMSC) Expansion Using RocketCell™ hMSC Xeno-Free Complete Growth Medium

A. Introduction

RocketCell™ hMSC Xeno-Free Complete Growth Medium (TheWell Bioscience, Cat #: RC06-GM) is an optimized defined growth factor containing phenol red-free expansion medium that provides the nutritional support necessary for the long-term expansion of undifferentiated human mesenchymal stem cells (hMSCs) grown on compatible extracellular matrix proteins, such as CytoGrow™ Recombinant Human Fibronectin (TheWell Bioscience, Cat #: CG024-C). It is recommended that hMSCs be expanded in a low-oxygen environment of 5% O_2 to optimize their growth kinetics and their phenotypes.

See Table #1 for sources of hMSCs successfully expanded using RocketCell™ hMSC Xeno-Free Complete Growth Medium, and Table #2 for immunophenotyping results.

The complete medium package includes a 500 mL bottle containing 485 mL of basal medium with a separate 15 mL vial of Supplement. It is recommended to use the RocketCell™ hMSC Xeno-Free Growth Medium together with the CytoGrow™ Recombinant Human Fibronectin.

It is recommended to use very gentle passaging methods as this will optimize the growth, longevity and stability of your hMSC cultures. We recommend using TrypLE™ cell dissociation reagent (ThermoFisher) or equivalent, as it's a plant derived recombinant xeno-free form of Trypsin, and is qualified to be gentle enough to be used with hESC/IPSCs cultures. Standard Trypsin/EDTA formulations are derived from animal sources, require Trypsin inhibitor neutralization, and often contain other enzymes which can be harmful to the long-term expansion of hMSCs.

For weekend-free culture technique, the following 7-day cycle is recommended.

a. Thaw/passage cells at low density (4000 cells/cm^2) on a Thursday*

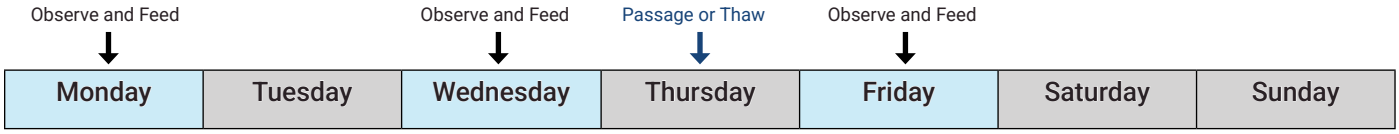
b. Verify culture integrity and feed on following Friday*

c. Feed on following Monday and Wednesday

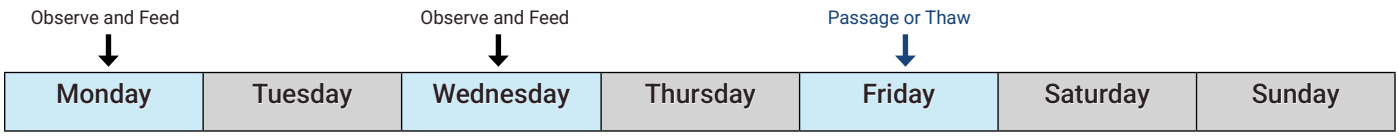
d. Passage on Thursday or Friday.

*If the user is experienced with established hMSC cultures, the above technique can be modified so that steps a and b are combined on Friday.

7 Day Cycle with Observation following Passaging or Thaw



7 Day Cycle following Passaging or Thaw



B. Materials and Reagents

1. RocketCell™ hMSC Xeno-Free Complete Growth Medium:

Components	Catalog No.	Size
RocketCell™ hMSC Xeno-Free Basal Medium	RC06-BM	485 mL
RocketCell™ hMSC Xeno-Free Supplement	RC06-S15	15 mL

2. Recommended companion product

Components	Catalog No.	Size
CytoGrow™ Recombinant Fibronectin (Human)	CG024-C	1 mg

3. Refer to Appendix A for additional cell culture medium and reagents from TheWell Bioscience.

C. Preparation of Medium and Supplement Component

NOTE: The frozen supplement should be defrosted at 4°C and used to supplement the Basal Medium. If the entire bottle of medium will not be used within two to four weeks, the supplement may be re-frozen at -20°C or -80°C in small aliquots in sterile o-ring screw top tubes and defrosted at 4°C as needed. Avoid repeated freeze-thaw cycles as growth factors will degrade.

Storage/Stability:

Components	Temperature	Time
RocketCell™ hMSC Xeno-Free Basal Medium	2°C-8°C, in the Dark	6 months
RocketCell™ hMSC Xeno-Free Complete Growth Medium with supplement	2°C-8°C, in the Dark	2 weeks
RocketCell™ hMSC Xeno-Free Supplement	-20°C to -80°C	1 year

Protocol

1. Defrost the 15 mL frozen RocketCell™ hMSC Xeno-Free Supplement at 4°C or on ice.
2. If the entire bottle of medium is used within 2 weeks, aseptically transfer the entire contents of the supplement into the basal medium. Otherwise, divide Supplement into aliquots and refreeze. **Do not freeze thaw supplement a second time.** The complete supplemented medium should be used within two weeks. Antibiotic/antimycotics agents may be added to the medium at the user's discretion.
3. Label the bottle with the date of preparation and the calculated expiration date (2 weeks from creation), and store at 2-8°C.

D. Preparing Complete Medium for Cell Culture

Protocol

1. Prior to cell culture, determine the volume of complete medium that will be required for feeding. It is generally recommended that the following volumes be used for the following standard flask sizes:

Flask Size	Growth Surface	Volume
T-12.5	12.5 cm^2	2 - 3 mL
T-25	25 cm^2	5 - 6 mL
T-75	75 cm^2	14 - 16 mL
T-225	225 cm^2	40 - 60 mL

2. Remove the calculated amount from the chilled medium into 50 mL conical tube and warm to approximately room temperature. Optionally, a 37°C bead bath (or water bath) may be used for more rapid warming.

E. Coating of Cell Culture Ware

NOTE: It is recommended that hMSCs be cultured on an appropriate extracellular matrix (ECM) substrate. In the human body, MSCs interact with ECM proteins through ***integrins*** displayed on their cell surfaces. Once activated, integrins propagate a variety of signals to the cellular nuclei, which may include survival as well as mitogenic (cell division) signals. While growth on uncoated cell culture plastic is possible, surfaces may not provide these critical cues as adhesion to uncoated surfaces is electrostatic, not integrin-mediated, and may not fully optimize the expected function of this growth media.

CytoGrow™ Recombinant Human Fibronectin was tested as an optimal coating substrate for RocketCell™ hMSC Xeno-Free Complete Growth Medium and promoted rapid growth rates.

Protocol

1. Reconstitute the CytoGrow™ Recombinant Human Fibronectin according to the product sheet instructions to 1 mg/mL using cold sterile cell culture grade water. Place the tube on ice or 4°C to complete solubilization.

2. Dilute the reconstituted fibronectin with cold Hanks Balanced Salt Solution dilution buffer 1:100 to 1:200 v/v to 5-10 µg/mL and coat the cell culture plates/dishes/flasks according to the following recommendations to ensure the bottom is covered completely by the fibronectin solution. **Do not allow the dish surface to dry out as this will result in a suboptimal performance.**

Flask Size	Surface Area	Recommnded Volume
T-12.5	12.5 cm^2	1 - 2 mL
T-25	25 cm^2	2 - 3 mL
T-75	75 cm^2	8 - 9 mL
T-225	225 cm^2	20 - 30 mL

Dish Size	Surface Area	Recommnded Volume
3.5 cm	12.5 cm^2	1 - 2 mL
6 cm	25 cm^2	2 - 3 mL
10 cm	75 cm^2	4 - 6 mL
15 cm	225 cm^2	10 - 15 mL

Plate Size	Surface Area/Well	Recommnded Volume/Well
48-well	0.95 cm^2	0.1 - 0.2 mL
24-well	1.9 cm^2	0.25 - 0.35 mL
12-well	3.8 cm^2	0.5 - 0.75 mL
6-well	9.5 cm^2	1 - 1.5 mL

3. Incubate the vessels at 37°C for 2 hours prior to use. Vessels that have been prepared in advance can be stored with vitronectin at 4°C for 1 week. Warm the stored vessels to 37°C for 30 min prior to use.

F. Thawing Cryopreserved hMSCs in RocketCell™ hMSC Xeno-Free Complete Growth Medium

NOTE: Thawing frozen cells is a rapid process wherein the entire ampoule is warmed at the same rate. This can be accomplished using a clean water bath set at 37°C, or with a thawing device such as a ThawStar™ (BioLife). If done correctly, thawing a single vial of cells frozen as a 1 mL aliquot should take approximately 2 minutes. It is critical that cell suspensions once thawed, be transferred into fresh cell culture medium (cool to room temperature) which dilutes the DMSO, a commonly used cryopreservative. We recommend freezing cell lines in 2 mL cryovials, as it leaves room for backfilling fresh medium into the cryovial prior to full transfer to larger volume for centrifugation. The example provided below is for plating cells onto a T75 fibronectin coated flask.

Protocol

1. Keep the vial of cells frozen in the vapor-phase nitrogen as most plastic cryovials are not rated for immersion in liquid phase. Vials can be transferred to dry ice for transport from the liquid nitrogen tank to the lab but not stored for more than a few hours outside LN_2 temperatures.
2. Prepare a 15 mL conical tube containing 9 mL of cold growth medium and set aside.
3. Precondition the ECM coated culture flasks using ½ the recommended volume of media. For example, aspirate the fibronectin coating from a T75, and add 7-8 mL of growth medium. Leave at room temperature in the laminar flow hood.
4. Remove cells from dry ice and place vial in a floater rack in a 37ºC water bath for 1.5 minutes. With ThawStar™, the vial is first sprayed with ethanol, and then placed into the device until thawed, then proceed to Step 8 below, otherwise proceed with Step 5.
5. Inspect the vial, and remove it from the 37ºC water bath when only a small piece of ice is still visible.

6. Carefully and quickly sanitize the exterior by generously spraying it with 70% ethanol, and transfer into sterile tissue culture hood.

7. Using sterile technique, wipe the excess ethanol, and remove the vial cap.

8. Take 1 mL of media from the 15 mL conical tube and gently add it to the 2 mL cryovial while stirring. This procedure is called back-fill. If this process is rushed, cells may experience osmotic shock, resulting in lower post-thaw viability.

9. Gently and slowly transfer the 2 mL back into the 8 mL remaining in the 15 mL conical tube.

10. Once the cells have been added to the medium, pellet them by centrifugation in a swinging bucket centrifuge at 100-120 x g for 5 minutes. Aspirate the supernatant, taking care not to disturb the cell pellet.

11. Resuspend the cells in 7-8 mL of fresh room temperature cell culture medium and count cell number.

12. It is recommended that cells be seeded at ≥4,000 viable cells/cm^2 (300,000 cells/T75 flask) although a slightly higher cell density (5,000-6,000 viable cells/cm^2) may be preferable for culture initiation as well as a higher cell passage number which may be exhibiting slower growth rates.

13. Add cells to the T-75 flask and return the flask into the incubator.

14. Inspect the following day and proceed to Section H.

G. Passaging Mesenchymal Stem Cells

NOTE: The end user should always be mindful that overexposure to digestive enzymes has the potential to damage cells or, in the case of pluripotent or multipotent stem cells, alter cellular phenotypes. Once cells have begun to "lift off" from the culture surface, gentle agitation of the flask by tapping may be employed to dislodge any weakly adherent cells. It is recommended that passaging be done with the TrypLE Cell Dissociation reagents (ThermoFisher) as experience has shown that they allow for gentler passaging, greater viability, and less disruption of cellular phenotypes. It is further recommended that if standard trypsin is used, that Trypsin Inhibitor be added following cellular collection to neutralize enzymes and terminate digestive activity.

At or with over-confluent cultures, the monolayer of hMSCs will produce noticeable extracellular matrix. This will appear as a loose gel when aspirating supernatant during routine media changes or passaging. This will not prevent passaging, however, while the cells may round up, they may remain attached to a web of extracellular matrix. Extended trituration may be needed to help dissociate the matrix.

Protocol (Example T75 flask)

1. Aspirate the medium from the flask.

2. Optional: rinse with 5 mL of PBS-EDTA. This will remove any dead cells, residual proteins and allow for quicker enzymatic digestion.

3. Add 3-5 mL TrypLE and incubate at 37°C for 5 min until cells begin to round up.

4. If cells have rounded up but **not** yet detached aspirate the TrypLE, and using 5 mL glass serological pipette, triturate cells off the flask surface with 5 mL of growth medium. Proceed to Step 6.

5. If cells **have** detached, triturate cells using a 5 mL glass serological pipette.

6. Transfer the dislodged cells to a 15 mL conical tube containing 5 mL of growth medium. The total volume is now 10 mL.

7. **Remove 10 µL and place in hemacytometer, and count cells in 2 to 3 quadrants.**

   a. **Example:** Average Cell Count = 100

   b. Multiply number by 10^4 to convert value to cells/mL = 1 million cells/mL.

   c. If sample **contains** TrypLE™ (Step 5 above) proceed to steps below, otherwise plate cells at desired density into new flasks.

   d. For T75 flask: If flask was preconditioned with 7-8 mL of medium, add 4000 cells/cm^2 = 300,000 cells, or 300 µL, and add an additional 7-8 mL of medium.

5. Centrifuge 15 ml conical tube at 100-120 x g for 5 min.

6. Carefully aspirate the supernatant (growth medium/TrypLE™), as not to disturb the pellet, and resuspend in 10 mL of growth medium so that original cell count determined in Step 7a-b is still accurate.

7. Plate as described above in Step 7d.

8. Return flask to cell culture incubator and proceed to Section H.

H. Migration to RocketCell™ hMSC Xeno-Free Complete Growth Medium

1. Cultures established and maintained in other media formulations may be migrated to RocketCell™ media either **acutely** or by stepwise titration during a one-week adaptation cycle.

2. For both types of adaptations, we recommend passaging cultures at 4000 cells/cm^2 in established medium on CytoGrow™ Fibronectin coated vessels and begin the media accommodation the following day. This allows the user to observe the behavior of the cells at low density and to determine if individual cells are forming colonies.

3. For **Acute** accommodation, change the entire media volume from the old media formulation to RocketCell™ hMSC one day post passage (Friday, in the 7-day cycle provided in Section A).

4. For **step-wise** accommodation using the 7-day cycle;

   - Change the medium to 75% old medium and 25% RocketCell™ hMSC one day post-passage (Friday);
   - 50% of each media on Monday;
   - 25% old and 75% RocketCell™ on Wednesday.
   - Passage on Thursday (or Friday) using 100% RocketCell™ hMSC Media.

I. Maintenance of hMSCs in RocketCell™ hMSC Xeno-Free Complete Growth Medium

1. hMSCs will adhere to the fibronectin coated dish within 1-2 hrs post plating. Observe the cultures the day following thawing (see Section F), or passaging (see Section G).

   - For beginners or when using unpredictable hMSC lines, we recommend passaging or thawing on Thursdays, so that one may verify success of the procedure on Friday before the feeding-free weekend.

2. Feed cells every 2 days by removing 80% of the media and replacing with fresh media as needed.

3. Once cultures reach near confluence, proceed with passaging in Section G above.

J. Freezing Mesenchymal Stem Cells

Introduction: Ideally, cryogenic freezing of any cell type should be conducted in a gradual, step-wise fashion, allowing the solution to uniformly cool throughout its volume. The use of a suitable cryoprotectant will ensure retention of cellular viability during storage. In general, DMSO-inclusive cryoprotectants are preferred for cellular storage. It has been experimentally determined that a DMSO concentration of 5% is adequate to preserve MSCs. Please check the concentration of DMSO in the freeze medium you normally use for cells as it may be 10% (1X) to 20% (2X) formulation. If your normal freeze medium contains 10% DMSO, you may proceed as normal or dilute with complete growth medium to 5%.

1. Passage and count cells as described above (see Section G).
2. Pellet cells by centrifugation as described but with centrifuge at 4°C.
3. Aspirate supernatant taking care not to disturb the cell pellet.
4. Resuspend cells in sufficient cool cryopreservative (final of 5% to 10% DMSO) to give a final concentration of 500,000-3,000,000 viable cells/mL, and place cells as 1 mL aliquots into 1.8-2 mL cryovials.
5. If possible, it is recommended that cells be frozen using a controlled-rate freezing unit with an optimized cooling protocol to gradually bring the final temperature to -80°C. If this is not possible, use of a "Mr. Frosty" cooling device (Nalgene) placed in a -80°C freezer.
6. Once vials have been frozen to -80°C, they should be transferred to liquid or vapor phase nitrogen storage. **Do not let vials remain at -80°C for more than 3 days.**

Tables

Table #1: Sources of hMSCs tested with RocketCell™ hMSC Xeno-Free Complete Growth Medium Media

Source	Name - Mesenchymal Stem Cells	Acronym	Growth
Liposaspirate	Adipose Derived	AD-MSC	positive
Bone Marrow aspirate	Bone Marrow Derived	BM-MSC	positive
Umbilical cord blood buffy coat	Umbilical Cord Blood	UCB-MSC	positive
Placental stroma	Placental Derived	PL-MSC	positive

Table #2: Immunophenotyping of MSC populations via immunofluorescence microscopy and flow cytometry

Marker	Bone-Marrow MSC	Umbilical Cord MSC	Placental MSC	Adipose-Derived MSC
CD13	Positive	Positive	Positive	Positive
CD14	Negative	Negative	Negative	Negative
CD19	Negative	Negative	Negative	Negative
CD29	Positive	Positive	Positive	Positive
CD31	Negative	Negative	Negative	Negative
CD34	Negative	Negative	Negative	Negative
CD44	Positive	Positive	Positive	Positive
CD45	Negative	Negative	Negative	Negative
CD73	Positive	Positive	Positive	Positive
CD90	**Positive**	**Positive**	**Positive**	**Positive**
CD105	**Positive**	**Positive**	**Positive**	**Positive**
CD146	Positive	Positive	Positive	Positive

Appendix A: Other products for culture of hMSCs

CASE STUDY

Expansion of hMSCs Cryopreserved for 20 Years in RocketCell™ hMSC Xeno-Free Complete Growth Medium

Materials

- **RocketCell™ hMSC Xeno-Free Complete Growth Medium (Catalog #: RC06-GM)**
- **VitroPrime™ Spread-Attach 24-well plate (Catalog #: VP-SA24W)**
- **CytoGrow™ Fibronectin (Catalog #: CG024-C)**
- **CytoGrow™ Vitronectin (Catalog #: CG082-C (CHO), Catalog #: CG083-C (HEK))**
- T75 Flask
- 24-well plate
- TrypLE™ (ThermoFisher, Cat#12604021)
- 50 mg/mL Gentamicin Sulfate (Med Chem Express, HY-A0276)

The Human Mesenchymal Stem Cell (hMSC) line was established from bone marrow from the hip of a male patient on September 15, 2005 using Knockout DMEM media (ThermoFisher, 10829018) supplemented with 10% FBS (Hyclone Defined, SH30070.03, Cytiva). The cultures were passaged 7 times, frozen on October 17, 2005, cryopreserved 3 days later into a Liquid Nitrogen in vapor phase tank (CryoExtra 20, ThermoFisher) and remained there until defrosted on July 25, 2025. Presently, all hMSC cultures were grown in low O_2 (6%) unless placed into an Incucyte S3 which operates at O_2 levels of approximately 20%.

The 15 mL supplement bottle of RocketCell™ hMSC Xeno-Free Complete Growth Media was defrosted overnight at 4°C. The following day, the supplement was mixed with the Basal Media, along with gentamicin sulfate (10 µg/mL). The vial of CytoGrow™ Fibronectin was removed from the freezer, and equilibrated to RT. The top was removed, and sterile cell culture grade water was added to resuspend the Fibronectin to a concentration of 1 mg/mL. The Fibronectin solution was diluted to 5 µg/mL using Hanks Balanced Salt solution and used to coat a T-75 flask and 24-well dish. A similar procedure was carried out for CytoGrow™ Vitronectin. The cultureware was placed into a humidified cell culture incubator for 1 hour prior to removing the coating solution and replacing with 10 mL for T-75, or 300 µL per well of the 24-well dish of RocketCell™ hMSC Xeno-Free Complete Growth Medium. The pre-conditioned dishes were returned to the incubator to equilibrate.

The ampoule of cells was removed from vapor phase LN_2, and placed onto dry ice. The tube was sprayed off with 70% ethanol, transferred into a biosafety cabinet, wiped off, and placed into a ThawStar tube defroster. After 2 min the tube was ejected, placed into a cryorack, the top removed, and 1 mL of cool hMSC media was back filled into the cryovial. The suspension was gently mixed, and the entire contents was slowly transferred into a 15 mL conical tube containing 8 mL of hMSC media. The tube was centrifuged at 100 x g for 5 min at 10°C. The supernatant was removed, and replaced with 1 mL of cool hMSC media. The pellet was resuspended using a P1000 pipette and then 50 µL was removed to a fresh 15 mL and diluted to 2.8 mL. The diluted cell suspension (100 µL/well) was then transferred to a fibronectin coated 24-well plate containing 300 µL of hMSC media per well. The remaining 950 uL was diluted with an additional 4 mL, and then transferred to a fibronectin coated T-75 flask with 10 mL of hMSC media. After 72 hours, the 24-well plate (passage 8) was placed into an IncuCyte S3, and 8 of the wells were scanned over the course of approximately 72 hours (Figure 1 & 2), then fixed with 4% paraformaldehyde to be set aside for immunostaining.

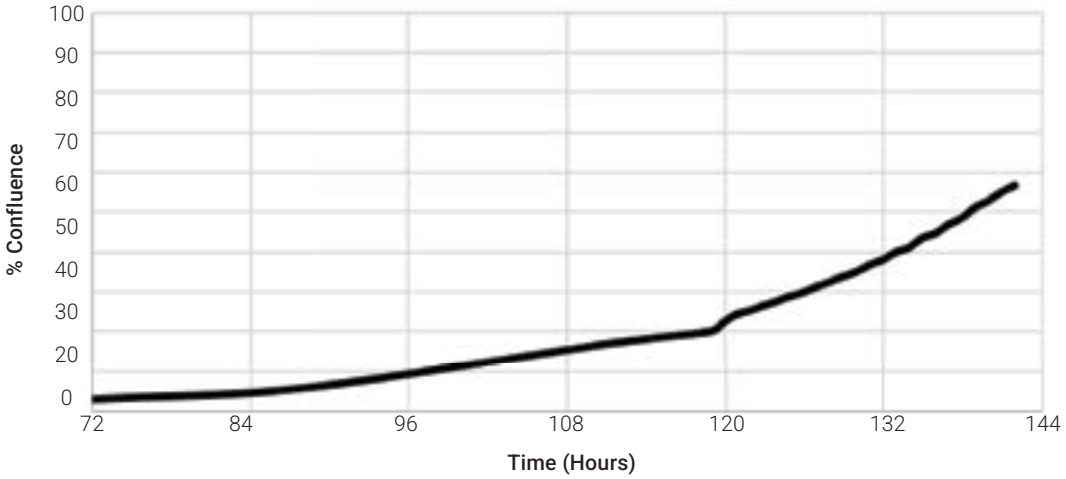


Figure 1: Growth of Cryopreserved hMSCs in RocketCell™ hMSC Xeno-Free Complete Growth Medium. Cells were defrosted after 20 years in liquid nitrogen storage, and placed into 24-well plate coated with fibronectin. Cultures were scanned using IncuCyte S3 72 hours after initial plating into low O_2 incubator. Similar cultures were expanded in fibronectin T-75 flask.

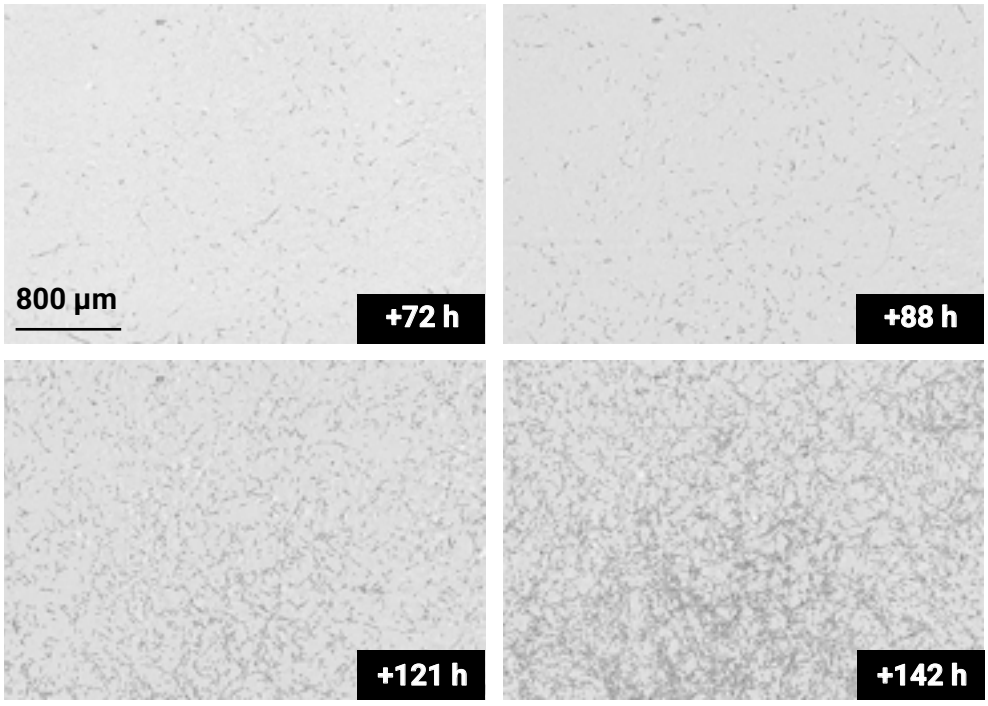


Figure 2: Phase contrast microscopy of thawed hMSCs: After 72 hrs in low O_2 incubator, a 24-well dish was transferred to an Incucyte S3, and scanned for an addition 142 hours.

After 7 days, the T-75 flask was passaged using TrypLE, and seeded (passage 9) into 2 fibronectin coated T-75 (375,000 and 1 million cells), and 10,000 cells per well into a 24-well plate. The 24-well plate was immediately placed into the Incucyte S3 and scanned for the following 7 days (Figure 3 & 4).

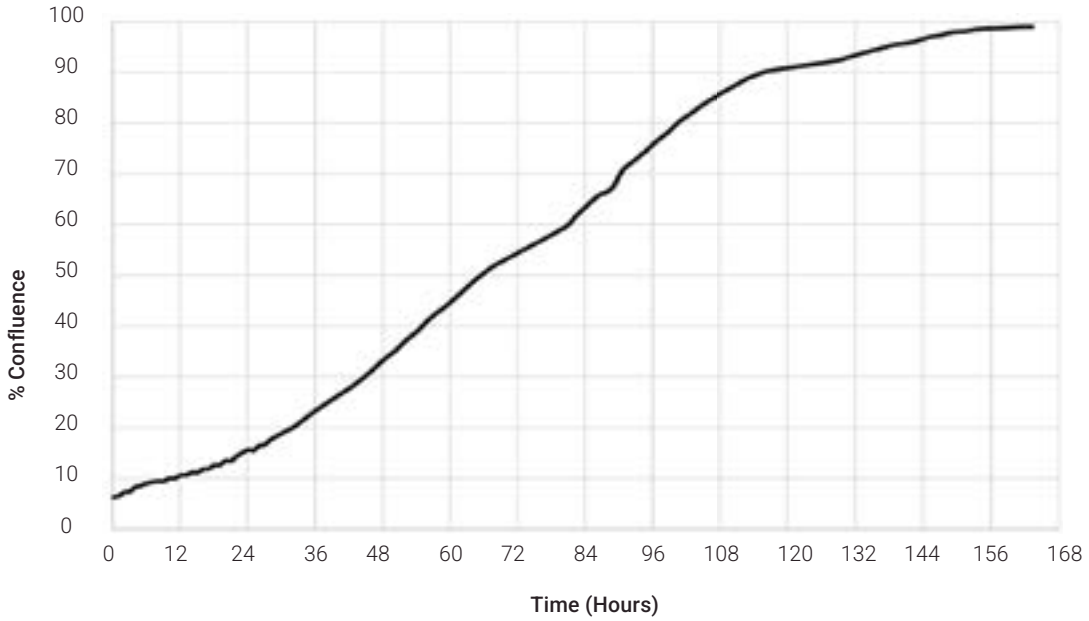


Figure 3: Growth of newly passaged hMSCs in RocketCell™ hMSC Xeno-Free Complete Growth Medium. Cells were passaged from a T-75 flask and plated into 24 well plate coated with fibronectin. Cultures were scanned using an IncuCyte S3.

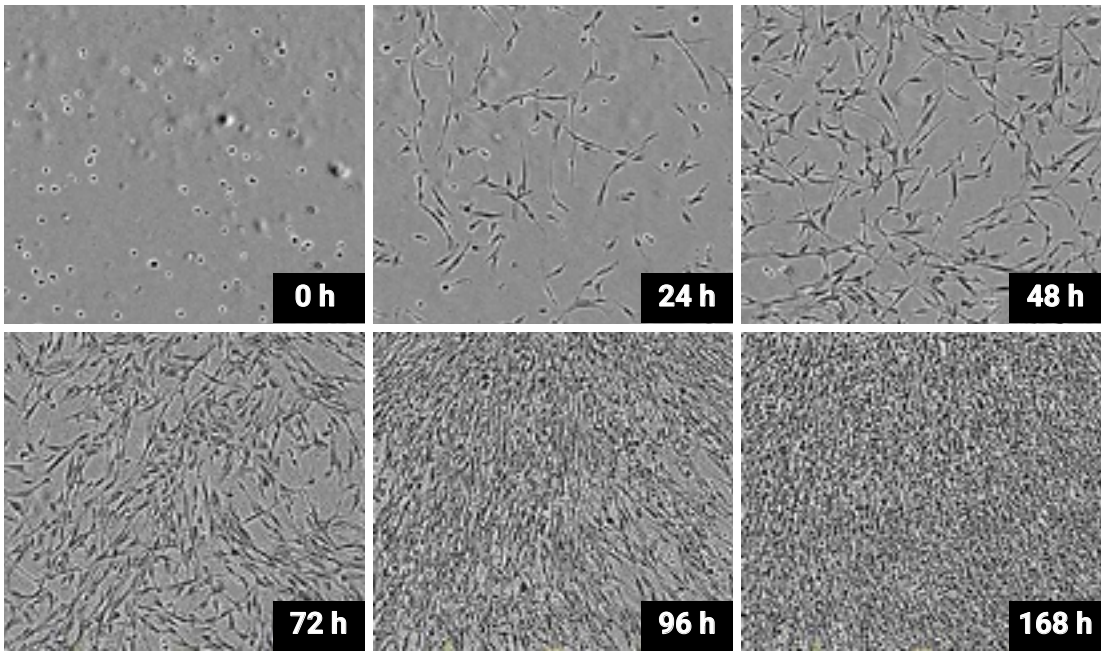


Figure 4: Phase contrast microscopy of newly passaged hMSCs: After passaging the 24-well plate was placed into an Incucyte S3, and scanned for up to 168 hrs. cale bar is 800 microns.

After 7 days, the 24-well plate was fixed with 4% paraformaldehyde and set aside for immunostaining. The T-75 flask (plated at 1 million cells/flask) was passaged using TrypLE and yielded 16.5 million cells. These cells were used to seed a 12-well VitroPrime™ Spread-Attach plate with 14,000 cells/well onto uncoated wells or those coated with CytoGrow™ Fibronectin, or CytoGrow™ Vitronectin. The dish was placed into an incubator and after 72 hours scanned using an Incucyte S3. (Figure 5).

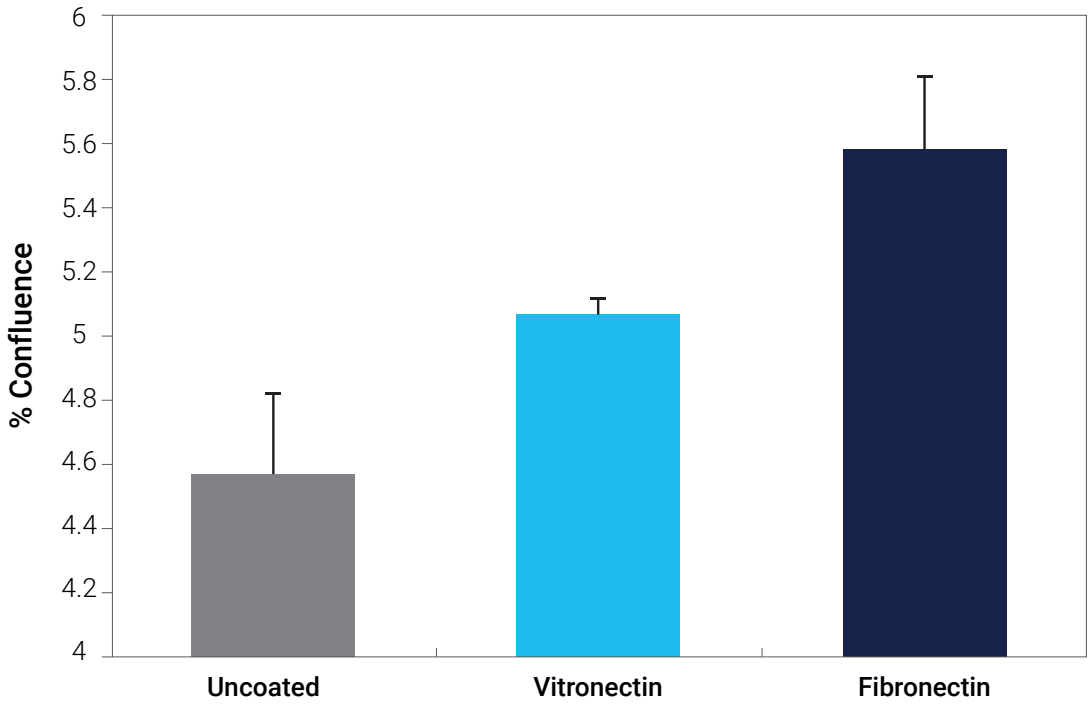


Figure 5: Growth of MSCs on VitroPrime™ Spread-Attach Surface. MSCs grown on standard TC treated plasticware were passaged, using TrypLE, and plated on a VitroPrime Spread-Attach 12-well plate on uncoated surface, or into wells coated with 5 µg/mL CytoGrow™ Vitronectin, or CytoGrow™ Fibronectin. The plates were placed into a low O_2 incubator and 72 hours later scanned using an IncuCyte S3, and data compiled from n=4 for each group.

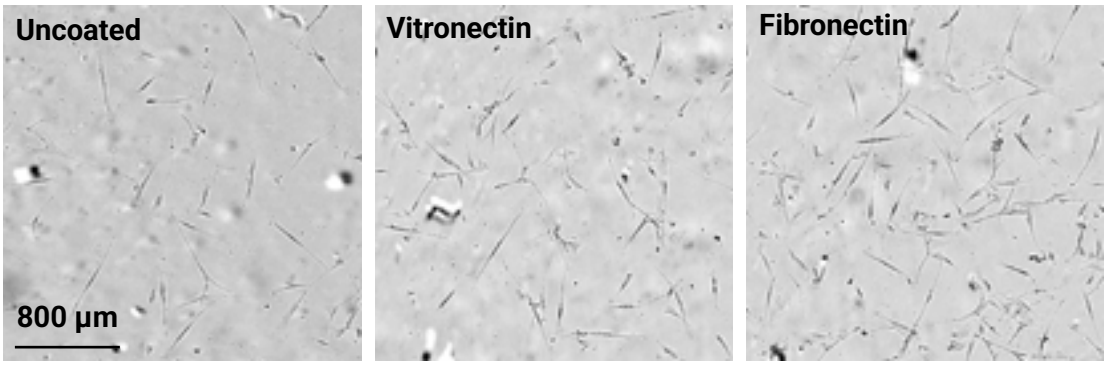


Figure 6: Growth of hMSCs on a matrix coated VitroPrime™ Spread-Attach plate. hMSCs passaged using TrypLE™ were plated at 14,000 cells/well using RocketCell™ hMSC Xeno-Free Complete Growth Medium and photographed 72 hours later.

Discussions

This case study demonstrated that a combination of RocketCell™ hMSC Xeno-Free Complete Growth Medium together with CytoGrow™ recombinant human Fibronectin as an extracellular matrix provided a robust system for the recovery and expansion of Human bone marrow derived mesenchymal stem cells that had been cryopreserved for 20 years. Further, we demonstrate that the VitroPrime™ Spread Attach surface alone is capable of sustaining hMSC growth, albeit more efficiently when coated with either xeno-free CytoGrow™ Vitronectin or the best with CytoGrow™ Fibronectin.

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