Instruction Manual of StemFit For Mesenchymal Stem Cell

Isolation of human MSC from bone-marrow and adipose tissue

1. Materials Required

-StemFit For MSC medium

-Synthemax II（Corning #3535）

-DPBS, no calcium, no magnesium（Life Technologies　#14190-144）

-TrypLE™ Select CTS™（Life Technologies　# A12859-01）

2. Media Preparation

Use sterile techniques to prepare StemFit For MSC medium.

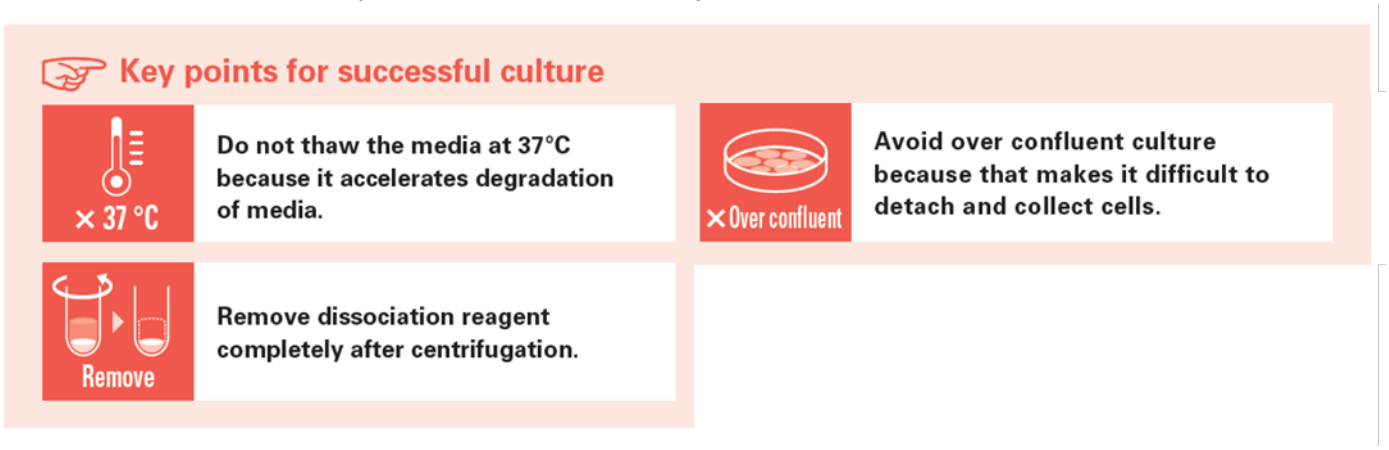
1. Thaw StemFit medium at room temperature (15-25 °C) or at 2-8°C overnight. Mix thoroughly. If precipitations are observed, keep the bottle at room temperature and dissolve them.

   ***CAUTION: Do not thaw StemFit For MSC at 37 °C**, as it accelerates medium degradation.*

   ＞ Thawed StemFit For MSC medium may be stored at 2-8 °C for up to a month. *Protect from light.*

   ＞ Optionally, the medium can be stored as aliquots at -20°C until the expiration date. Do not re-freeze thawed aliquots.

2. Warm medium to room temperature and use immediately.



3. Isolation Protocol (Bone marrow)

■ **Culture plate coating**

Coat the plate / dish with Synthemax II (5 µg/cm2) as follows. See the manufacture's protocol for details (https://www.corning.com/catalog/cls/documents/protocols/protocol_CLS_AN_204_Synthemax_Substrate-Self-Coating.pdf).

1. Dilute Corning Synthemax II-SC stock solution (1 mg/mL) 1:40 in cell culture grade water to achieve a 0.025 mg/mL working solution final concentration.
2. Add appropriate volume of Corning Synthemax II-SC working solution to a culture vessel.

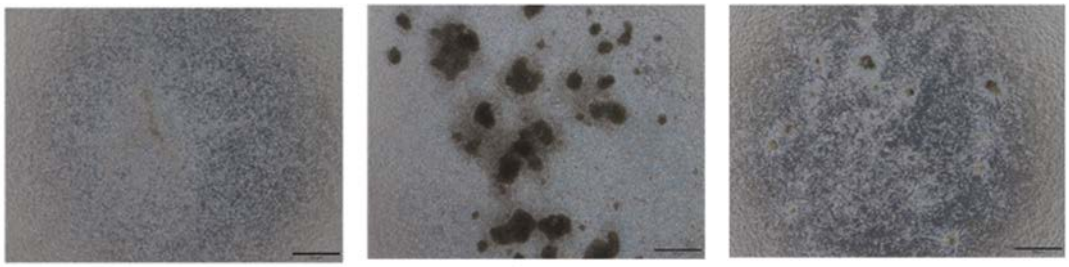
   ＞ **Example**: 2 mL / well for 6 well plate.　＞ **Example**: 15 mL / flask for T75 flask.
3. Cover vessel with lid and incubate at room temperature for 2 hours.

4. Aspirate all remaining solution (vessels will appear to be dry).

   CAUTION: Do not coat the plate / dish with other ECMs (e.g. iMatrix-511) instead of Synthemax II since it drastically decreases the performance of isolation.

■ Isolation

1. Obtain fresh mononuclear cells (MNC) from human bone marrow (BM) via density centrifugation (Ficoll-Paque), or thaw the frozen BMMNC. Determine the cell concentration.
2. Seed BMMNC at 1.3 x 10^6 cells / cm^2 in Synthemax II coated plate/dish with StemFit For MSC medium.
   ＞Example: 2.6×10^6 cells / 0.5 mL / well in 24 well plate.
   ＞Example: 1.3×10^7 cells / 2 mL / well in 6 well plate.
   ＞Example: 2.7×10^7 cells / 5 mL / dish in 60 mm dish.
3. Culture the cells at 37℃,5% CO_2.
4. Change the medium the next day.
5. Add 20% medium on day 3 and 5 after seeding.
6. 7 days after seeding, transfer all the cells to a new culture vessel according to the procedure in 7-14.



7. Prepare "StemFit For MSC + Synthemax medium" by adding Synthemax II-SC stock solution (1 mg/mL) to StemFit For MSC medium to a final concentration of 1 μg/mL.
   ＞Example: Add 10 μL of 1 mg/mL Synthemax II-SC stock solution into 10 mL StemFit For MSC.
8. Collect the culture supernatant in a polypropylene (PP) conical tube.
9. Add DPBS to the culture vessel and pipette the cells 10 times to fully dissociate cells and transfer them to the PP conical tube prepared in step 8.
   ＞Example: 0.5 mL DPBS / well in 24 well plate.
   ＞Example: 2 mL DPBS / well in 6 well plate.
   ＞Example: 5 mL DPBS / dish in 60 mm dish.
10. To collect cells remaining in the vessel, add DPBS to the culture vessel and transfer it to the PP conical tube.
11. Centrifuge at 200 ×g for 5 min at room temperature. Decelerate without the use of an applied brake.
12. Aspirate the supernatant completely.
13. Tap the tube to loosen the pellet and resuspend the cells with "StemFit For MSC + Synthemax medium".
    ＞Example: 0.5 mL / well in 24 well plate.
    ＞Example: 2 mL / well in 6 well plate.
    ＞Example: 5 mL / dish in 60 mm dish.
14. Seed all the cells in a new culture vessel.
15. Change the medium 3 days after replating. After that, change the medium once in every 2-3 days.
16. Subculture when cells are approximately 70-90% confluent.

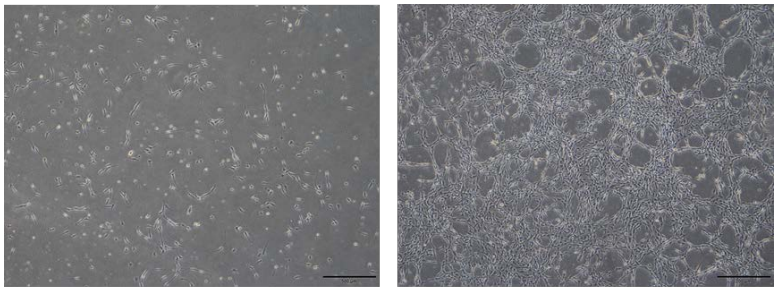
4. Isolation Protocol (Addipose tissue)

■ **Culture plate coating**

Coat the plate / dish with Synthemax II (5 µg/cm^2) as shown in chapter 3.

■ **Isolation**

1. Obtain fresh adipose tissue and process with collagenase. Determine the cell concentration.
2. Seed cells at 2-6 x 10^3 cells / cm^2 in Synthemax II coated plate/dish with StemFit For MSC medium.
3. Culture the cells at 37℃,5% CO^2 for 5-7 days until 70-90% confluent without a medium change.
4. Subculture when cells are approximately 70-90% confluent.

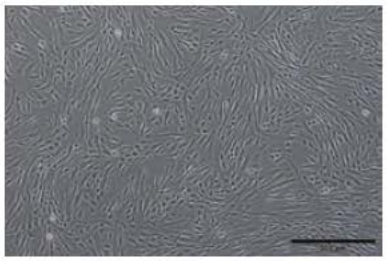


Day 4 Day 7

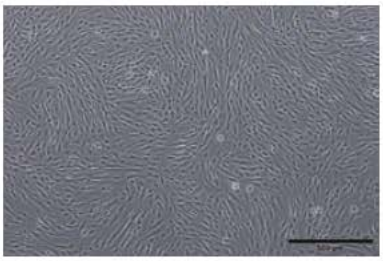
5. Passage

1. Prepare “StemFit For MSC + Synthemax medium” by adding Synthemax II-SC stock solution (1 mg/mL) to StemFit For MSC medium to a final concentration of 1 µg/mL.
   ＞ **Example**: Add 10 µL of 1 mg/mL Synthemax II-SC stock solution into 10 mL StemFit For MSC.
2. Aspirate the medium and wash once with DPBS.
3. Add Cell Detachment Solution [e.g. TrypLE™ Select (Thermo Fisher) or Accumax (MERCK Millipore)]
   ＞ **Example**: 500 µL / well for 6 well plate. ＞ **Example**: 4 mL / flask for T75 flask.
4. Incubate at 37°C for 10 mins until all cells are rounded and the dissociation of cells is apparent.
5. Pipette the cells in the Cell Detachment Solution to fully dissociate cells and transfer to a PP conical tube.
6. To collect cells remaining in the vessel, add DPBS to the well / flask and then transfer to the conical tube.
   ＞ **Example**: 1 mL / well for 6 well plate. ＞ **Example**: 8 mL / flask for T75 flask.
7. Centrifuge at 200 x g for 5 min at room temperature.
8. Aspirate the supernatant completely.
   Caution: Eliminate dissociation reagent completely. Remaining dissociation reagent may inhibit cell attachment to culture vessel.
9. Tap the tube to loosen the pellet and resuspend the cells with 0.5-1 mL “StemFit For MSC + Synthemax medium”.
   Note: Please adjust the volume of medium according to the culture scale.
10. Determine the cell concentration.
11. Seed the cells at 5.0 x 10^3 cells / cm^2 in “StemFit For MSC + Synthemax medium”.
    ＞ **Example**: 5.0 x 10^4 cells / 2 mL / well in 6 well plate. ＞ **Example**: 3.8 x 10^5 cells / 10-15 mL in T75 flask.
12. Culture the cells at 37°C, 5% CO_2.
13. Change the medium once in 2-3 days.
14. Subculture when cells are approximately 70-90% confluent.
    Caution: Do not allow cells to become over confluent since it will be difficult to detach and collect

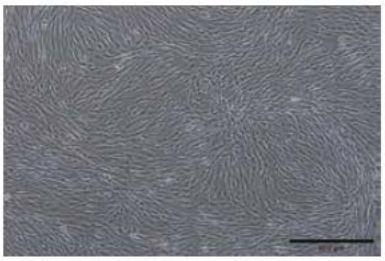
cells. Passage should be done while cells are between conditions shown in (a) and (b).



(a) 70% confluent



(b) 90% confluent



(c) over confluent

6. FAQs & Troubleshooting

- **From which tissues can StemFit For MSC medium be used for isolation?**
  - StemFit for MSC medium can be used for isolation MSCs from bone marrow and adipose tissue. Please contact us directly for other tissues.
- **Cells cannot attach to the plate after seeding**
  - Please check if Synthemax II coated plate is used for isolation. StemFit For MSC medium does not contain ECM.
  - Please check if Synthemax II is added to the medium for passage. StemFit For MSC medium does not contain ECM.
  - Please check if dissociation reagent was completely eliminated after centrifugation. Remaining dissociation reagent inhibits attachment.
- **Cells cannot detach from the plate during passage**
  - Please check if cells are not more than 90% confluent. When cells become overly confluent, it can be difficult to make single cells. Please passage your cells at 70-90% confluent.
- **Cells do not become single cells after dissociation**
  - Please check if cells are not more than 90% confluent. When cells become overly confluent, it can be difficult to make single cells. Please passage your cells at 70-90% confluent.
  - Please pipette the cells in the Cell Detachment Solution to fully dissociate cells. Please also refer to step. No.5 of chapter “5. Passage.”

7. Precaution and disclaimer

This isolation method is patent pending (Patent Application No. 2019-156537, 2020-012333).

8. Contact information

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