**Please cite this article as:** Benedetti, A. *et al.* (2021). A Simple Method for the Isolation and *in vitro* Expansion of Highly Pure Mouse and Human Satellite Cells. *Bio-protocol* 11(23): e4238. DOI: 10.21769/BioProtoc.4238.

bio-protocol www.bio-protocol.org/e4238 Bio-protocol 11(23): e4238. DOI:10.21769/BioProtoc.4238

# A Simple Method for the Isolation and *in vitro* Expansion of Highly Pure Mouse and Human Satellite Cells

Anna Benedetti[1], Gianluca Cera[2, 3], Daniele De Meo[2, 3], Ciro Villani[2, 3], Marina Bouche[1] and Biliana Lozanoska-Ochser[1, *]

[1]Department of Anatomical, Histological, Forensic and Orthopedic Sciences, Section of Histology and Embryology, Sapienza University of Rome, Rome, Italy

[2]Department of Anatomical, Histological, Forensic and Orthopedic Sciences, Section of Orthopedics, Sapienza University of Rome, Rome, Italy

[3]Department of Orthopaedics and Traumatology, Policlinico Umberto I, Rome, Italy

*For correspondence: biliana.lozanoska-ochser@uniroma1.it

**[Abstract]** Satellite cells (SCs) are muscle stem cells capable of regenerating injured muscle. The study of their functional potential depends on the availability of methods for the isolation and expansion of pure SCs, which retain myogenic properties after serial passages *in vitro*. Here, we describe a protocol for the isolation and *in vitro* expansion of highly pure mouse and human SCs based on ice-cold treatment (ICT). The ICT is carried out by briefly incubating the dish containing a heterogeneous mix of adherent muscle mononuclear cells on ice for 15-30 min, which leads to the detachment only of the SCs, and gives rise to SC cultures with 95-100% purity. This approach can also be used to passage the cells, allowing SC expansion over extended periods of time without compromising their proliferation or differentiation potential. Overall, the ICT method is cost-effective, accessible, technically simple, reproducible, and highly efficient.

**Graphic abstract:**

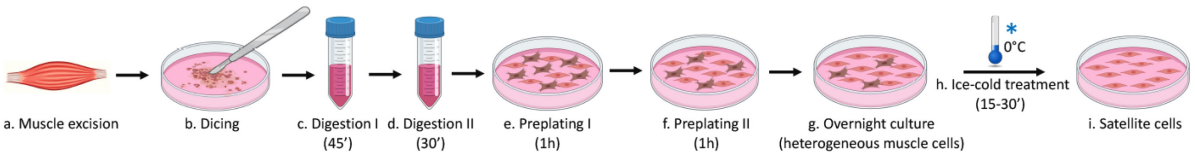


**Figure 1. Satellite cell isolation using the ice-cold treatment method.**

**Keywords:** Satellite cell isolation, *In vitro* expansion, Skeletal muscle, Pax7, MyoD

**[Background]** The exceptional regenerative ability of skeletal muscle is primarily due to a resident population of stem cells called satellite cells (SCs) (Mauro, 1961; Chang and Rudnicki, 2014; Wang *et al.*, 2014). The study of their functional potential depends on the availability of methods for the isolation and expansion of highly pure SCs with preserved myogenic properties after serial passages *in vitro* (Danoviz and Yablonka-Reuveni, 2012; Keire *et al.*, 2013; Syverud *et al.*, 2014).

Presently, there are three main methods commonly used for the isolation of SCs: pre-plating, fluorescence activated cell sorting (FACS), and magnetic bead isolation.

The pre-plating method is based on the differing adhesive properties of muscle cells, with SCs being the least adherent (Gharaibeh *et al.*, 2008; Danoviz and Yablonka-Reuveni, 2012; Keire *et al.*, 2013; Syverud *et al.*, 2014). Although cheap and straightforward to perform, this method's main disadvantages are that it is time consuming and gives rise to cultures of variable purity, often with fibroblast contamination and overgrowth by day 7 of culture (Keire *et al.*, 2013).

The FACS sorting method sorts muscle mononuclear cells pre-labeled with SC specific antibodies (Fukada *et al.*, 2004; Sherwood *et al.*, 2004; Montarras *et al.*, 2005; Pasut *et al.*, 2012; Chapman *et al.*, 2013; Liu *et al.*, 2015). At present, FACS sorting is the gold standard for the isolation and study of SCs. Nevertheless, there are several disadvantages to this method, including high cost and the requirement for a FACS sorter instrument. Moreover, this method is time consuming, requires expertise to perform, and cell purity can be variable. The cell labeling step that precedes the sorting procedure can potentially stress or damage the cells, decrease their viability, or alter their functional properties *in vitro* (Syverud *et al.*, 2014).

Finally, the third method is based on magnetic cell separation (MACS) and uses magnetic columns and SC specific magnetic bead kits (Blanco-Bose *et al.*, 2001). Because this method assumes that all the other cell types are successfully removed from the muscle cell preparation, it is less precise than the FACS sorting method. This method is expensive to perform, time consuming, and stressful for the cells. As for the other two methods, cell purity is variable, and often the SC cultures become overgrown by fibroblasts by day 7 (Keire *et al.*, 2013; Syverud *et al.*, 2014).

The ideal SC isolation technique would permit isolation of pure SCs with minimal manipulation, producing cells that could be expanded *ex vivo* without losing their stemness and regenerative capacity. Here, we describe a protocol for ice-cold treatment (ICT); this is a simple, inexpensive, and efficient method for the isolation and long-term expansion of highly pure mouse and human SCs that preserves their myogenic potential (Benedetti *et al.*, 2020). In terms of purity of the isolated cell population, the ICT method outperforms others, such as pre-plating or magnetic bead isolation. Furthermore, it is fast and easy to perform—apart from the time required for enzymatic digestion (1.5 h), it involves minimal manipulation of the cells. Another major advantage of the ICT method is that it doubles up as a very gentle passaging technique, allowing long-term serial expansion of SCs *ex vivo* without altering their proliferation and differentiation properties. In turn, this drastically reduces the number of mice or muscle biopsies required to obtain a sufficient number of cells (Benedetti *et al.*, 2021). The ICT method permits growing mouse and human SCs to be passaged at least 10 times, expanding their number 150- and 300-fold, respectively (Benedetti *et al.*, 2021). This represents a clear advantage over the most commonly used passaging reagent (trypsin), which typically accelerates the differentiation of passaged SCs after only two passages (Danoviz and Yablonka-Reuveni, 2012; Benedetti *et al.*, 2020 and 2021; Fiore *et al.*, 2020).

Overall, the cost-effectiveness, accessibility, and technical simplicity of this protocol, as well as its remarkable efficiency, represent major improvements over existing protocols. The next step will be to test this protocol for the isolation and expansion of stem cells from tissues other than muscle.

## Materials and Reagents

1. Scalpel and surgical blades (Securelab, size 24)
2. 70 µm cell strainers (Falcon, catalog number, catalog number: 352350)
3. 40 µm cell strainers (Falcon, catalog number, catalog number: 352340)
4. 100 mm tissue culture dishes (Falcon, catalog number: 430167)
5. 35 mm tissue culture dishes (Falcon, catalog number: 353001)
6. 60 mm tissue culture dishes (Falcon, catalog number: 353002)
7. 50 ml polypropylene centrifuge tubes (Falcon, catalog number: 352098)
8. 10 ml serological pipettes (Falcon, catalog number: 357551)
9. Dissection boards (Styrofoam board)
10. Cover slips 24 × 50 (Menzel, catalog number: 15737592)
11. Pipette tips (Corning)
12. Parafilm (Sigma, catalog number: P7793)
13. WT C57BL/6J mice aged 4-6 weeks (The Jackson Laboratory)
14. Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, catalog number: D5671)
15. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A7030)
16. Hydrophobic PAP pen for immunostaining (Sigma-Aldrich, catalog number: Z377821)
17. Human muscle biopsies [*i.e.*, gluteus maximus biopsies obtained from patients undergoing surgery; Benedetti *et al.* (2021)]
18. Vectashield mounting medium (Vector Laboratories, catalog number: H-1000-10)
19. Hoechst 33342 staining dye (Abcam, catalog number: ab228551)
20. Primary antibodies:
    Pax7 (Developmental Studies Hybridoma Bank)
    MyoD (Santa Cruz Biotechnology, catalog number: sc-760)
    MyoG (F5D) (Developmental Studies Hybridoma Bank)
    MyHC (MF20) (Developmental Studies Hybridoma Bank)
21. Fluorescently labeled secondary antibodies:
    Goat anti-rabbit Alexa Fluor 488 (1:1,000, Abcam, catalog number: 150077)
    Goat anti-mouse Alexa Fluor 555 (1:1,000, Thermo Fisher Scientific, catalog number: A28180)
22. 0.1% gelatin (Stem Cell Technologies, catalog number: 07903)
23. L-glutamine (Sigma-Aldrich, catalog number: 59202C)
24. Chicken embryo extract (Seralab, catalog number: CE-650-J)
25. Goat serum (Sigma-Aldrich, catalog number: G9023)
26. Horse serum (Thermo Fisher Scientific, catalog number: 26050088)

27. Fetal bovine serum (FBS) (Sigma-Aldrich, catalog number: F2442), heat inactivated at 56°C for 30 min
28. Dulbecco's Phosphate-Buffered Saline (PBS) with $MgCl_2$ and $CaCl_2$ (Sigma-Aldrich, catalog number: D8662)
29. Dulbecco's Phosphate-Buffered Saline (PBS), modified without $MgCl_2$ and $CaCl_2$ (Sigma-Aldrich, catalog number: D8537)
30. Penicillin-streptomycin (pen/strep) solution (Sigma-Aldrich, catalog number: P0781)
31. Gentamycin solution (Sigma-Aldrich, catalog number: G1397)
32. Formaldehyde solution 4% (Sigma-Aldrich, catalog number: 1004968350)
33. Methanol (Sigma-Aldrich, catalog number: 34860)
34. Collagenase type II (Sigma-Aldrich, catalog number: C6885)
35. Collagenase/dispase (Roche, catalog number: 11097113001)
36. Ethanol (Sigma-Aldrich, catalog number: 51976)
37. 70% ethanol (see Recipes)
38. Collagenase type II digestion solution (see Recipes)
39. Collagenase/dispase digestion solution (see Recipes)
40. Growth medium (see Recipes)
41. Differentiation medium (see Recipes)
42. Neutralization buffer (see Recipes)
43. Hoechst solution (see Recipes)

## Equipment

1. Microsurgery scissors (Fine Science Tool, catalog number: 14184-09)
2. Microsurgery tweezers (Fine Science Tool, catalog number: 11252-00)
3. Pipettes (Gilson, P10, 20, 200, 1000)
4. Humidified chamber (prepared by wetting paper towels with distilled water and placing them in a plastic container with a lid)
5. Centrifuge (Eppendorf, model: 5702)
6. Vertical Autoclave (Falc, model: ATV80)
7. Temperature regulated shaking water bath (GLS, catalog number: 1083)
8. Biosafety cabinet (Gelaire, model: BSB4 A)
9. Laboratory chemical fume hood (ESCO, Frontier Acela)
10. $CO_2$ incubator Thermo Forma (Thermo Fisher Scientific, model: 3110)
11. Zeiss Axioskop 2 Plus microscope (Carl Zeiss)
12. Phase-contrast microscope (Nikon Eclipse, model: TS100)
13. Pipet Controllers for serological pipettes (Falcon)
14. Hemocytometer counting chamber Neubauer improved (BLAUBRAND, model: BR717810)
15. Ice machine

## Software

1. ZEISS ZEN 2 Blue edition (Carl Zeiss) (downloa from https://www.zeiss.com/microscopy/int/products/microscope-software.html)
2. ImageJ 1.53a (download from https://imagej.nih.gov/ij/download.html)

## Procedure

A. Isolation of muscle satellite cells

*Notes:*

*a. Experimental procedures should be performed only after obtaining ethical approval.*

*b. Follow the same procedure for both mouse and human muscle.*

**Isolation of mouse satellite cells**

1. Day 1: Preparation prior to muscle dissection
   a. Prepare 70% ethanol (see Recipe).
   b. Autoclave dissection instruments (scissors and tweezers).
   c. Add 3 ml of DMEM supplemented with 1% pen/strep into 60 mm cell culture dishes (one dish/mouse or one dish per gram of human muscle biopsy). These will be used to collect the dissected muscle.
   d. Coat the cell culture dishes with 0.1% gelatin:
      Coat 35 mm and 100 mm cell culture dishes with 1 ml and 5 ml of 0.1% gelatin, respectively, ensuring that the entire surface of the dish is evenly coated, and incubate for 30 min in a cell incubator at 37°C with 5% $CO_2$. Next, remove excess gelatin (without washing the dishes), and leave the dishes open to air dry for 10-15 min under the hood. These dishes will be used for cell culture.
      *Note: The 35 mm dishes will be needed one day following isolation.*
   e. Prepare collagenase type II and collagenase/dispase digestion solutions (see Recipes).
   f. Prepare enzyme neutralization buffer (see Recipes).
   g. Prepare cell growth medium (see Recipes).
   h. Prepare cell differentiation medium (see Recipes).
   i. Sterilize a scalpel and surgical blades.
   j. Pre-warm a water bath at 37°C.
2. Day 1: Dissection of mouse hindlimb muscles
   For a schematic representation of the dissection process, see Figure 2.

**Please cite this article as:** Benedetti, A. *et al.* (2021). A Simple Method for the Isolation and *in vitro* Expansion of Highly Pure Mouse and Human Satellite Cells. *Bio-protocol* 11(23): e4238. DOI: 10.21769/BioProtoc.4238.



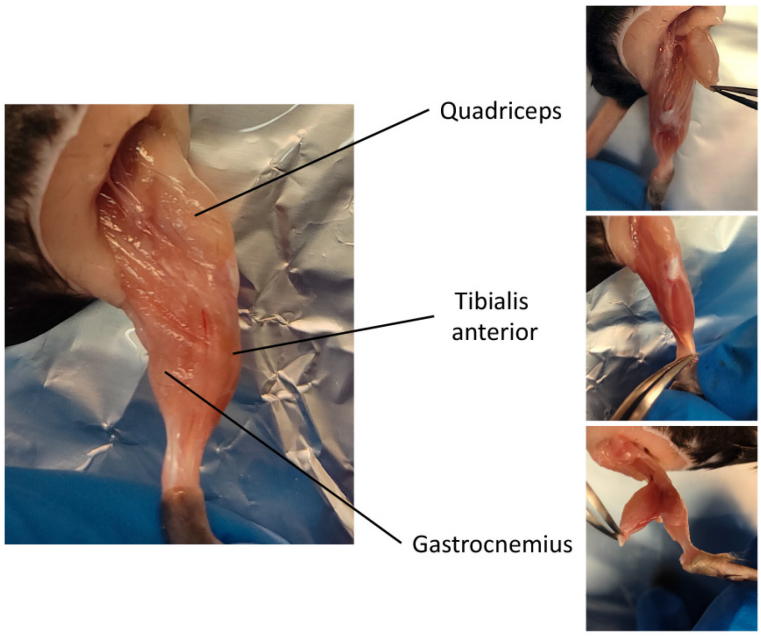


**Figure 2. Dissection of mouse quadriceps, tibialis anterior, and gastrocnemius muscles.**

a. Euthanize a mouse [*i.e.*, by cervical dislocation; Benedetti *et al*. (2021)].
b. Pin the mouse to a dissection board and spray it with 70% ethanol.
c. Using dissection scissors, make an incision on the skin above the ankle, and peel off the skin over the entire length of the hindlimbs to expose the muscles below.
d. Collect the tibialis anterior, quadriceps, and gastrocnemius muscles from both legs (Figure 2).
e. Place the dissected muscles in a 60 mm dish containing DMEM supplemented with 1% pen/strep, and remove any hair to minimize the risk of contamination.
f. Transfer the dishes under a biosafety cabinet and finely cut up the muscles into 1 mm pieces using a sterile scalpel (Figure 1b).
   *Note: Remove and discard any fat from the dissected muscle.*
g. Transfer the minced muscles into a 50 ml tube and centrifuge at 300 × *g* for 5 min at room temperature (RT).

3. Day 1: Muscle digestion I (Figure 1c).
   a. Aspirate the supernatant with a 10 ml serological pipette and resuspend the minced muscles in 10 ml of collagenase type II digestion solution per gram of tissue (see Recipes).
      *Note: It is important to use a pipette to remove the supernatant, as the pellet is not stable in this step.*
   b. Seal the tubes with parafilm and transfer them to a shaking (100 rpm) pre-warmed (37°C) water bath for 45 min.
      *Note: Position the tubes horizontally so that they are completely submerged in water.*
   c. Spray the tubes with 70% ethanol and transfer them to a biosafety cabinet.

 

d. Block the digestion enzyme by adding 10 ml of enzyme neutralization buffer per gram of tissue (see Recipes).
e. Pipette up and down with a 10 ml serological pipette five times.
f. Centrifuge at 300 × *g* for 5 min at RT.

4. Day 1: Muscle digestion II (Figure 1d).
   a. Aspirate the supernatant with a 10 ml serological pipette and resuspend the pellet in 10 ml of collagenase/dispase digestion solution per gram of tissue (see Recipes).
   b. Seal the tubes with parafilm and transfer them into a shaking (100 rpm) pre-warmed (37°C) water bath (as in Step A3b) for 30 min.
   c. Spray the tubes with 70% ethanol and transfer them to a biosafety cabinet.
   d. Block the digestion by adding 10 ml of enzyme neutralization buffer per gram of tissue (see Recipes).
   e. Pipette up and down with a 10 ml serological pipette five times.
   f. Sieve the digested muscles through a 70 µm cell strainer into a new 50 ml tube.
   g. Sieve the digested muscles through a 40 µm cell strainer into a new 50 ml tube.
      *Note: An adequately digested muscle will pass through the strainers easily. If necessary, the muscle digest can be passed again through another strainer to get rid of most of the cell debris and undigested muscle.*
   h. Centrifuge the tube with muscle digest at 300 × *g* for 5 min at RT.
   i. Discard the supernatant and resuspend the pellet in 10 ml of DMEM 10% FBS. Count the number of muscle mononuclear cells using trypan blue.
      *Note: At this step, you can combine the pellets if more than 1 mouse was used. On average, the number of cells released after digestion from 3, 4-8-weeks-old mice is around $10^7$ cells.*

5. Day 1: Pre-plating step (Figure 1e-1f).
   a. Plate the cells at $10^5$/ml in a 100 mm dish (uncoated) in 20 ml of DMEM 10% FBS 1% pen/strep and incubate at 37°C for 1 h.
   b. Collect the non-adhered cells in a 50 ml tube and centrifuge at 300 × *g* for 5 min at RT.
   c. Discard the supernatant and resuspend the cell pellet in DMEM 10% FBS 1% pen/strep.
   d. Count the resuspended cells and plate them at $10^5$/ml into a 100 mm dish (uncoated) in 20 ml of DMEM 10% FBS 1% pen/strep. Incubate the cells at 37°C for 1 h (as in Step A5a).
   e. Collect and transfer the non-adhered cells into a 50 ml tube.
      *Note: On average, the pre-plating steps remove around 50% of strongly adherent cells such as fibroblasts.*
   f. Centrifuge the tube with non-adhered cells at 300 × *g* for 5 min at RT.
   g. Discard the supernatant and resuspend the cell pellet in growth medium (see Recipes).
   h. Plate the cells into 0.1% gelatin coated 100 mm dishes at $10^6$ cells/dish. Grow them overnight (Figure 1g).

6. Day 2: Ice-cold treatment (ICT) (Figure 1h).
   a. Wash the dishes containing the heterogeneous mix of adhered muscle cells three times with

 

10 ml of PBS (with $MgCl_2$ and $CaCl_2$) at RT.

*Note: Gently pipette PBS all around the dish.*

b. Add 10 ml of ice-cold PBS (without $MgCl_2$ and $CaCl_2$) to each 100 mm dish.

*Note: Both PBS with or without $CaCl_2$ can be used. However, using PBS without $CaCl_2$ will facilitate detachment.*

c. Fill a polystyrene box with ice (0°C) and place the dishes on top of the ice for 15-30 min with occasional gentle manual shaking of the dish (creating a swirling motion).

*Notes:*

*i. If purity is of utmost importance (e.g., 99-100% purity), the dishes should not be kept on ice for longer than 30 min. Nevertheless, the dishes can be kept on ice for up to 1 h to increase cell yield, with purity still high at 90%.*

*ii. The original dishes (from day 1 of the isolation procedure) containing heterogeneous muscle cells can be kept up to day 3 of culture and used for further rounds of ICTs .*

d. Collect the detached cells into a 50 ml tube.

e. Wash the original dish once more with 10 ml of PBS to collect the remaining detached cells.

*Note: Gently pipette PBS all around the dish.*

f. Centrifuge the cells at 300 × *g* for 5 min at RT.

g. Discard the supernatant and resuspend the cells in growth medium.

h. Count the cells.

*Note: On average, 1.5 × $10^4$-2 × $10^4$ SCs can be collected after 30 min on ice at 24 h of culture of the original dish. A further 4 × $10^4$ SCs can be collected after the second or third ICT at 48 h or 72 h of culture).*

i. Plate the cells into 35 mm dishes precoated with 0.1% gelatin, at a density of $10^3$ cells/dish and place them into a cell incubator.

*Note: At this density, the SC proliferate for up to three days. Afterward, they begin to differentiate. Increasing the plating density will accelerate the time it takes for the myoblasts to differentiate and fuse into myotubes.*

7. Cell culture

a. Culture the cells in growth medium, changing the medium every two days.

b. To induce myoblast differentiation, remove growth medium and replace it with differentiation medium (see Recipes) once the cells reach 80% confluency.

8. Cell passaging

a. Place the dishes containing proliferating SCs on ice for up to 30 min.

b. Follow the steps described in A6.

**Isolation of human muscle satellite cells**

*Notes:*

*a. Experimental procedures using human muscle biopsies should be performed only after obtaining ethical approval and informed consent.*

*b. The number of SCs obtained from human muscle biopsies may vary depending on the age of the donor, with a smaller number of cells typically obtained from older donors.*

1. Preparation prior to muscle dissection
   Proceed as in Step A1.
2. Muscle cutting
   a. Place the human muscle biopsy in a 60 mm dish containing DMEM supplemented with 1% pen/strep. Work in a biosafety cabinet.
   b. Remove any fat and connective tissue using scissors.
   c. Finely cut up the muscle into 1 mm pieces using a sterile scalpel.
   d. Transfer the minced muscles to a 50 ml tube and centrifuge at 300 × *g* for 5 min.
3. Muscle digestion I:
   Proceed as described in A3.
4. Muscle digestion II:
   Proceed as described in A4.
   *Note: On average, 2 × $10^6$ muscle mononuclear cells can be obtained from digestion of 1 g of human muscle biopsy.*
5. Pre-plating:
   Proceed as in A5.
6. ICT:
   Proceed as in A6.
   To further enrich in SCs the heterogeneous muscle cell culture on day 1 after isolation, it may be necessary to grow it for an additional 3-4 days before proceeding to ICT (when the culture reaches a confluency of about 80%).
   *Notes:*
   *a. On average, 1 × $10^4$ SCs can be collected after the first ICT.*
   *b. A further 2 × $10^4$ SCs can be collected after the second or third ICT at 48 h or 72 h of culture.*
7. Cell culture
   Proceed as in Step A7.
   *Note: Human SCs proliferate slower than mouse SCs, with a doubling time of 46 h.*

B. Analysis of mouse and human satellite cell specific marker expression by immunofluorescence staining
   1. Grow the cells in a 35 mm dish.
   2. Aspirate the medium and wash the dishes once with 1 ml of PBS with $MgCl_2$ and $CaCl_2$.
   3. Fix the cells by adding 4% PFA (1 ml/35 mm dish) and incubate for 10 min at room temperature.
      *Note: PFA is toxic and must be used under a chemical fume hood.*
   4. Discard the PFA into a toxic waste container and wash three times with 1 ml of PBS for 5 min each.

5. Permeabilize the cells by adding cold methanol (1 ml/35 mm dish) and incubate at -20°C for 6 min.
6. Discard the methanol into a toxic waste container and wash three times with 1 ml of PBS for 5 min each.
7. Remove the PBS and mark the border of the dish with a hydrophobic pen.
8. Block by adding PBS containing 5% goat serum (100 µl/35 mm dish) for 30 min at RT.
9. Remove the blocking solution and incubate the cells with the primary antibodies (100 µl/35 mm dish) at the appropriate dilution [Pax7 (1:10), MyoD (1:50), Myogenin (1:20), and Myosin Heavy Chain (1:20) diluted in sterile filtered 4% BSA/PBS] overnight in a humidified chamber at 4°C.
10. Remove the primary antibody solution and wash three times with 1 ml of PBS for 5 min each.
11. Incubate the cells with the secondary antibodies (100 µl/35 mm dish) [goat anti-rabbit Alexa Fluor 488 (1:1,000) and goat anti-mouse Alexa Fluor 555 (1:1,000) diluted in sterile filtered 1%BSA/PBS] for 1h at room temperature in a humified chamber in the dark.
12. Remove the secondary antibody solution and wash three times with 1 ml of PBS for 5 min each.
13. Counterstain the nuclei with Hoechst solution (see Recipes) (100 µl/35 mm dish) for 5 min at room temperature in the dark.
14. Remove the Hoechst solution and wash three times with 1 ml of PBS for 5 min each.
15. Mount the coverslips with Vectashield mounting medium (30 µl/35 mm dish).
16. Analyze under a fluorescence microscope.

## Data analysis

Cell morphology was analyzed under a bright field microscope (Figures 3A and 4A). To analyze the expression of myogenic markers such as Pax7, MyoD, Myogenin, and Myosin Heavy Chain (MHC), the cells were stained as described in the procedure section (Figure 3B and 3D). Mouse SC purity was above 99% after ICT isolation. SC purity was assessed by immunofluorescence staining for Pax7 and MyoD, by counting the percentage of SCs positive for Pax7 and/or MyoD at day 3 of culture in growth medium after ICT (Figure 3B and 3C) (Benedetti *et al*., 2021).

The purity of human SCs isolated using the ICT approach is typically above 97% after ICT. Because human SCs in culture rapidly downregulate the expression of Pax7, SC purity was assessed by immunofluorescence staining for myogenin, by counting the percentage of SCs positive for myogenin at day 5 of culture in growth medium (Figure 4B and 4C) (Benedetti *et al*., 2021). The differentiation potential of human SCs was analyzed by performing immunofluorescence staining with an anti-Myosin Heavy Chain (MHC) antibody (Figure 4D).

**Please cite this article as:** Benedetti, A. *et al.* (2021). A Simple Method for the Isolation and *in vitro* Expansion of Highly Pure Mouse and Human Satellite Cells. *Bio-protocol* 11(23): e4238. DOI: 10.21769/BioProtoc.4238.



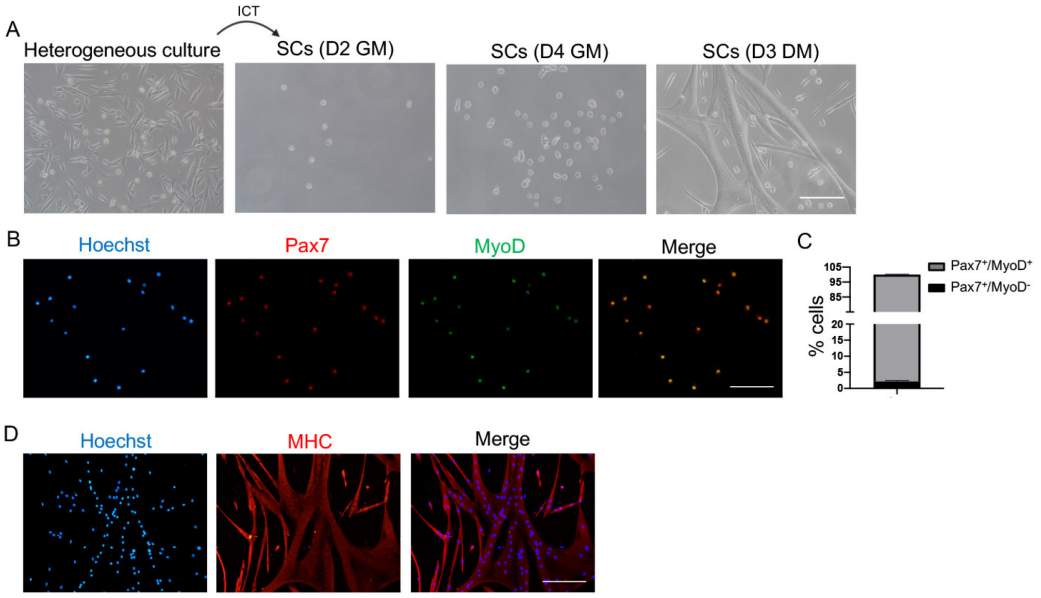


**Figure 3. Isolation and characterization of mouse muscle SCs using the ICT method.**

**A.** Representative bright field images of the mouse muscle cell culture. SCs were isolated by ICT from heterogeneous mononuclear cells. The ICT-isolated SCs at day 2 (D2) and day 4 (D4) in growth medium (GM) and at day 3 (D3) after adding differentiation medium (DM). **B.** Representative immunofluorescence images of ICT-isolated SCs stained for Pax7 (red), MyoD (green), and nuclei (blue). **C.** Graph showing the percentage of cells positive for Pax7 and/or MyoD at D3 of culture in growth medium after ICT (n = 3 independent experiments). **D.** Representative immunofluorescence images of ICT-isolated SCs stained for myosin heavy chain (MHC) (red) and nuclei (blue) after four days in growth medium followed by three days in DM. Scale bar = 100 µm. Error bars represent mean ± SEM (Benedetti *et al.*, 2021).

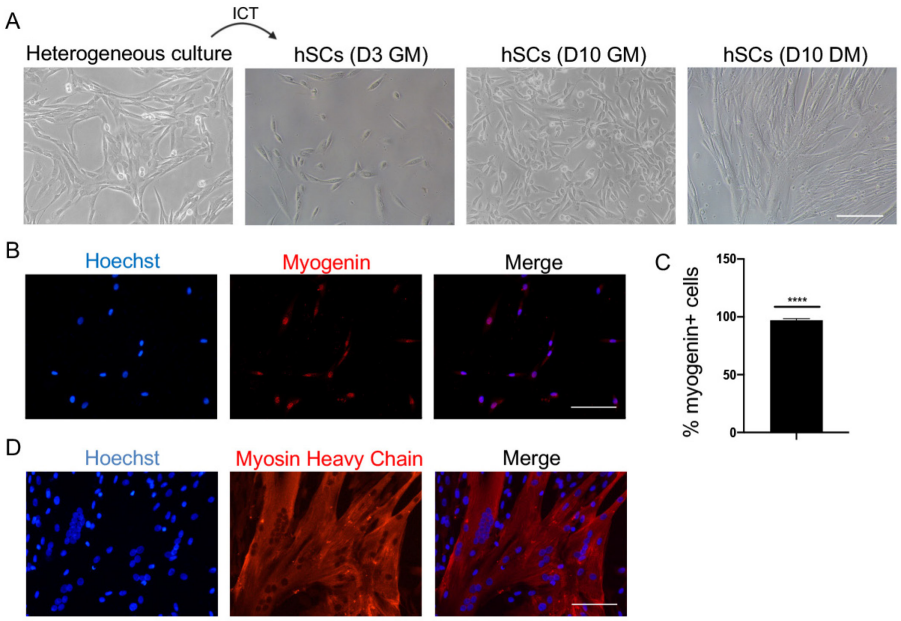


**Figure 4. Isolation and characterization of human muscle SCs (hSCs) using the ICT method.**

**A.** Representative bright field images of the heterogeneous human muscle mononuclear cell culture and isolated SCs at day 3 (D3) and day 10 (D10) in growth medium (GM), and at D10 after adding differentiation medium (DM). **B.** Representative immunofluorescence images of human SCs stained for Myogenin (red) and nuclei (blue). **C.** Graph showing the percentage of myogenin+ cells after ICT at day 5 of culture in GM (n = 3 independent experiments). **D.** Representative immunofluorescence images of human SCs stained for myosin heavy chain (MHC) (red) and nuclei (blue) after 10 days in GM followed by 10 days in DM. Scale bar = 100 µm. Error bars represent mean ± SEM (Benedetti *et al.*, 2021).

## Recipes

1. 70% ethanol
   Add 70 ml of 100% ethanol to 30 ml of dd$H_2O$
   Store at room temperature
2. Collagenase type II digestion solution
   Dissolve collagenase type II powder in PBS with $MgCl_2$ and $CaCl_2$ at a concentration of 0.4 mg/ml, for a total of 10 ml per gram of tissue.
3. Collagenase/dispase digestion solution
   Dissolve collagenase/dispase powder in PBS $MgCl_2$ and $CaCl_2$ free at a concentration of 1 mg/ml, for a total of 10 ml for gram of tissue.
4. Growth medium
   Supplement DMEM medium with 20% horse serum, 3% chicken embryo extract, 1% penicillin/streptomycin, and 1% L-Glutamine. Store at 4°C.
5. Differentiation medium
   Supplement DMEM medium with 5% horse serum, 1% chicken embryo extract, 1% penicillin/streptomycin, and 1% L-Glutamine. Store at 4°C.
6. Neutralisation buffer
   Supplement DMEM medium with 10% FBS and 1% penicillin/ streptomicin.
7. Hoechst solution
   Dilute Hoechst stain 1:1,000 in PBS

## Acknowledgments

This work was supported by a grant from The Dutch Duchenne Parent Project NL (DPP NL) to BLO; research grants from Parent Project Italy (PP, Italy) to MB and from the University of Rome (RP11715C7D238352, RM118164275C7EBE and RM11916B7E20311C to MB, and AR11715C7F9E158E, AR11816436905518 and AR11916B7E2A7B64 to AB). This protocol describes the work published in Benedetti *et al.* (2021).

 

## Competing interests

The authors declare no competing financial or non-financial interests.

## Ethics

All procedures involving mice were approved by the Italian Ministry for Health and were conducted according to the EU regulations and the Italian Law on Animal Research. Ethical Approval ID: 82945.19. valid until 18/01/2023.

All patients gave informed consent to undergo intra-operative muscle biopsy and to publish the clinical and laboratory data obtained.

## References

1. Benedetti, A., Fiore, P. F., Madaro, L., Lozanoska-Ochser, B. and Bouche, M. (2020). Targeting PKCtheta Promotes Satellite Cell Self-Renewal. *Int J Mol Sci* 21(7): 2419.
2. Benedetti, A., Cera, G., De Meo, D., Villani, C., Bouche, M. and Lozanoska-Ochser, B. (2021). A novel approach for the isolation and long-term expansion of pure satellite cells based on ice-cold treatment. *Skelet Muscle* 11(1): 7.
3. Blanco-Bose, W. E., Yao, C. C., Kramer, R. H. and Blau, H. M. (2001). Purification of mouse primary myoblasts based on α7 integrin expression. *Exp Cell Res* 265(2): 212-220.
4. Chang, N. C. and Rudnicki, M. A. (2014). Satellite cells: the architects of skeletal muscle. *Curr Top Dev Biol* 107: 161-181.
5. Chapman, M. R., Balakrishnan, K. R., Li, J., Conboy, M. J., Huang, H., Mohanty, S. K., Jabart, E., Hack, J., Conboy, I. M. and Sohn, L. L. (2013). Sorting single satellite cells from individual myofibers reveals heterogeneity in cell-surface markers and myogenic capacity. *Integr Biol (Camb)* 5(4): 692-702.
6. Danoviz, M. E. and Yablonka-Reuveni, Z. (2012). Skeletal muscle satellite cells: background and methods for isolation and analysis in a primary culture system. *Methods Mol Biol* 798: 21-52.
7. Fiore, P. F., Benedetti, A., Sandona, M., Madaro, L., De Bardi, M., Saccone, V., Puri, P. L., Gargioli, C., Lozanoska-Ochser, B. and Bouche, M. (2020). Lack of PKCtheta Promotes Regenerative Ability of Muscle Stem Cells in Chronic Muscle Injury. *Int J Mol Sci* 21(3) : 932.
8. Fukada, S., Higuchi, S., Segawa, M., Koda, K., Yamamoto, Y., Tsujikawa, K., Kohama, Y., Uezumi, A., Imamura, M., Miyagoe-Suzuki, Y. *et al.* (2004). Purification and cell-surface marker characterization of quiescent satellite cells from murine skeletal muscle by a novel monoclonal antibody. *Exp Cell Res* 296(2): 245-255.
9. Gharaibeh, B., Lu, A., Tebbets, J., Zheng, B., Feduska, J., Crisan, M., Peault, B., Cummins, J. and Huard, J. (2008). Isolation of a slowly adhering cell fraction containing stem cells from

 

murine skeletal muscle by the preplate technique. *Nat Protoc* 3(9): 1501-1509.

10. Keire, P., Shearer, A., Shefer, G. and Yablonka-Reuveni, Z. (2013). Isolation and culture of skeletal muscle myofibers as a means to analyze satellite cells. *Methods Mol Biol* 946: 431-468.
11. Liu, L., Cheung, T. H., Charville, G. W. and Rando, T. A. (2015). Isolation of skeletal muscle stem cells by fluorescence-activated cell sorting. *Nat Protoc* 10(10): 1612-1624.
12. Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol* 9: 493-495.
13. Montarras, D., Morgan, J., Collins, C., Relaix, F., Zaffran, S., Cumano, A., Partridge, T. and Buckingham, M. (2005). Direct isolation of satellite cells for skeletal muscle regeneration. *Science* 309(5743): 2064-2067.
14. Pasut, A., Oleynik, P. and Rudnicki, M. A. (2012). Isolation of muscle stem cells by fluorescence activated cell sorting cytometry. *Methods Mol Biol* 798: 53-64.
15. Sherwood, R. I., Christensen, J. L., Conboy, I. M., Conboy, M. J., Rando, T. A., Weissman, I. L. and Wagers, A. J. (2004). Isolation of adult mouse myogenic progenitors: functional heterogeneity of cells within and engrafting skeletal muscle. *Cell* 119(4): 543-554.
16. Syverud, B. C., Lee, J. D., VanDusen, K. W. and Larkin, L. M. (2014). Isolation and Purification of Satellite Cells for Skeletal Muscle Tissue Engineering. *J Regen Med* 3(2).
17. Wang, Y. X., Dumont, N. A. and Rudnicki, M. A. (2014). Muscle stem cells at a glance. *J Cell Sci* 127(Pt 21): 4543-4548.

 