

Rat Calvariae Osteoblast Cells

Instructions for use

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I. Receiving instructions: Unpack immediately! Packages may contain components with various storage requirements!

II. Safety

THESE PRODUCTS ARE FOR RESEARCH USE ONLY. Not approved for human or veterinary use, for application to humans or animals, or for use *in vitro* diagnostic or clinical procedures. **WARNING:** Handle as a potentially biohazardous material under biosafety level 1 containment. These cells are not known to contain an agent known to cause disease in healthy adult humans. These cells have not been screened for hepatitis B, human immunodeficiency viruses or other adventitious agents. If you require further information, please contact your site safety officer or scientific support.

III. Unpacking and storage instructions

1. Cells should be stored in liquid nitrogen. Do NOT store cells at -80°C. The cells are extremely temperature-sensitive and should be transferred to liquid nitrogen **immediately** upon arrival. Cells should be transported on dry ice or in a liquid nitrogen container. When transporting the cells on dry ice make sure that the vials are **completely** covered.
2. Upon arrival, store basal media at 4°C, protected from the light. Store SingleQuots™ Supplement at -20°C in a freezer that is not self-defrosting. Once the media is supplemented with growth factors (SingleQuots™ Supplement) it may be stored for up to 4 weeks at 4°C.

IV. Preparation of medium

The recommended media for the Rat Calvariae Osteoblasts is DMEM with 4.5 g/L glucose (Lonza No. 12-604F) supplemented with the MSCGM™ hMSC SingleQuots™ Supplements (catalog number PT-4105) for proliferation studies and hMSC Osteogenic Differentiation SingleQuots™ Kit (catalog number PT-4120) for mineralization studies. **See below for exact details on preparing media.**

Growth medium:

1. Thaw the SingleQuots™ Medium and FBS at room temperature.
2. Decontaminate the external surfaces of all supplement vials and the medium bottle with ethanol or isopropanol.
3. Aseptically open the FBS, L-glutamine, and GA-1000 vials and add the entire amount to 500 mL of DMEM with a pipette.
4. Rinse the empty vials with medium. It may not be possible to recover the entire volume listed, but the small losses will not affect the cell growth characteristics of the medium.

Differentiation medium:

NOTE: One (1) hMSC Osteogenic SingleQuots™ Kit is sufficient for supplementing 170 mL media (for total of 200 mL differentiation medium). More than one hMSC Osteogenic SingleQuots™ Kit may be needed to prepare enough media for entire differentiation period.

1. Thaw the SingleQuots™ Medium and FBS at room temperature.
2. Decontaminate the external surfaces of all supplement vials and the medium bottle with ethanol or isopropanol.
3. Aseptically open the FBS, L-glutamine, GA-1000, ascorbate, dexamethasone, and β-glycerophosphate vials and add entire amounts to 170 mL of DMEM with a pipette.
4. Rinse the empty vials with medium. It may not be possible to recover the entire volume listed, but the small losses will not affect the cell growth characteristics of the medium.

5. Depending on the number of multi-well plates set up for mineralization, more than 200 mL of differentiation media may be needed.
For example, if 2-6 well plates are seeded with cells for mineralization:
 - a. 3 mL of differentiation media per well is needed.
 - b. 12 wells total x 3 mL/well = 36 mL differentiation media.
 - c. Recommend media changes 2x per week: 36 mL x 2 media changes/week = 72 mL differentiation media per week.
 - d. Cells require approximately 3-4 weeks for mineralization: ~75 mL per week x 3-4 weeks = 225 mL-300 mL differentiation media for entire mineralization period.
6. It is recommended to aliquot the differentiation medium into amounts needed for each media change. Warm only the number of aliquots needed for a media change to room temperature. Store the remaining aliquots at 2-8°C until use.

V. Thawing of cells / initiation of culture process

1. Remove a vial of cells from liquid nitrogen and place in a water bath pre-heated to 37°C. Important: do not centrifuge or vortex the cells. Keep the time between removing the vial from liquid nitrogen tank and placing into the preheated water bath as short as possible.
2. After 2 minutes, remove the vial and disinfect the outside by wiping with 70% ethanol. Work in a laminar flow hood. Proceed with the next step immediately after thawing.
3. Gently transfer 0.25 mL of cells into a 50 mL centrifuge tube and immediately add 4.75 mL pre-warmed medium containing 10% FBS drop-wise onto cells, while rotating the tube by hand.
4. Mix the cell suspension by carefully pipetting. **Important:** do not centrifuge or vortex the cells.
5. Count cells using trypan blue 0.4% solution (Lonza No. 17-942E).
6. **For mineralization studies**, plate cells at 7,000 cells/mL (or 21,000 cells/well) per well of 6-well plate in 3 mL growth media. One vial contains enough cells for plating approximately three 6-well plates. Other

multi-well plate formats can be utilized and seeding density should be adjusted accordingly.

NOTE: For optimal mineralization, cells should not be passaged prior to plating.

7. **For proliferation studies**, plate cells at 5,000 cells/cm² in flasks with the appropriate amount of growth media. One vial contains enough cells for plating three T-25 flasks in 5 mL growth media per flask or one T-75 flask in 15 mL growth media.
8. Incubate the cells overnight at 37°C in 5% CO₂ incubator.
9. Next day change the medium to remove any residual DMSO on cells plated in either flasks or multi-well plates. Add the appropriate amount of fresh, pre-warmed growth medium. Avoid repeated warming and cooling of the medium. If the entire contents are not needed for a single procedure, transfer only the required volume to a sterile secondary container.
10. For flasks, incubate at 37°C with 5% CO₂ changing medium two times per week or every 3-4 days. When cells appear confluent they can be trypsinized and re-plated.
11. For multi-well plates, incubate at 37°C with 5% CO₂ until cells have reached at least 80% confluence, which could require 2-3 days.
12. Upon reaching appropriate confluence, induce differentiation and mineralization by removing growth medium and replacing with differentiation medium.
13. Continue to change differentiation medium two times per week until cells have mineralized (approximately 3-4 weeks).
14. Mineralization can be assessed a variety of ways, although it is recommended to use the OsteoImage™ Mineralization Assay (Lonza No. PA-1503). See Figure 1.

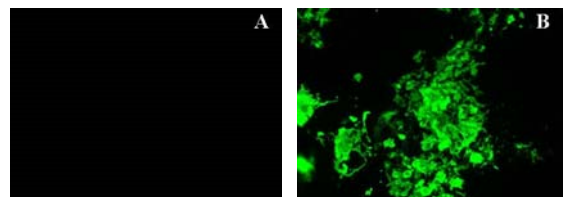


Figure 1. Rat calvariae osteoblasts were plated at 21,000 cells/well in a 6-well plated and cultured in either growth medium as a control (A) or differentiation medium (B) for 21 days. Mineralization was assessed using the OsteoImage™ Mineralization Assay.

VI. Subculture instructions:

The following instructions are for a T-25 flask. Adjust all volumes accordingly for other size flasks.

Preparation for subculturing

1. Subculture the cells when they are 80-90% confluent.
2. For each 25 cm² of cells to be subcultured:
 - a. Thaw 2 mL of trypsin-Versene[®] and allow to come to room temperature.
 - b. Allow 5 mL of HEPES buffered saline solution (HEPES-BSS) to come to room temperature.
 - c. Allow 4 mL of trypsin neutralizing solution (TNS) to come to room temperature.
 - d. Remove the growth medium from 4°C storage and allow to warm to room temperature.

Work in a laminar flow hood

1. Prepare T-25 culture flasks for inoculation by adding 5 mL growth medium and equilibrate them in the incubator at 37°C, 5% CO₂ for 30 min before inoculating the cells.
2. Aspirate medium from the growth flask and add 5 mL HEPES-BSS to rinse the cells.
3. Aspirate HEPES-BSS from the flask and cover the cells in the flask with 2 mL trypsin-Versene[®] (EDTA) mixture 1X solution 17-161.
4. Rock the flask to make sure all cells come into contact with the trypsin-Versene[®].
5. Tighten the cap and place the flask into the incubator.
6. Remove the flask after 2 minutes and check for cell detachment under the microscope, if majority of cells are not detached, return to incubator and check cells every two minutes until 90% of the cells are rounded up (see step 7). Do not allow trypsin to stay on the monolayer longer than 10 minutes.
7. When most of the cells are rounded up, tap the flask against the palm of your hand to release the majority of the cells from the culture flask. If only a few cells detach, you may not have let them trypsinize long enough. Wait 30 seconds and tap again. Repeat the procedure until 90% of cells are detached. Do not try to get all cells detached by tapping them severely. This action may damage the cells.
8. After cells are released, neutralize the trypsin in the flask with 4 mL TNS solution.
9. Transfer the cell suspension to a 15 mL conical tube.

10. Centrifuge the cells at 300 x g for 5 minutes.
11. Perform a cell count and calculate the total number of cells and the volume of cell suspension needed to inoculate the flasks.
12. Dispense the calculated volume into prepared subculture flasks.

VII. Ordering information

When placing an order or for scientific support, please refer to the product numbers and descriptions listed above. For a complete listing of all these products, refer to the Lonza website or our current catalog. To obtain a catalog, additional information or scientific support you may contact Lonza by web, e-mail, telephone, fax or mail.

Cat. No.	Product	Description
R-OST-583	Rat Calvariae Osteoblasts	≥ 0.5 million cells in a 0.25 mL cell suspension
12-604F	DMEM, High Glucose	500 mL
PT-4105	MSCGM™ hMSC SingleQuots™ Kit	1 kit
PT-4120	hMSC Osteogenic Differentiation SingleQuots™ Kit	1 kit
CC-5022	HEPES	100 mL
17-161E	Trypsin-Versene [®] mixture	100 mL
CC-5002	Trypsin neutralizing solution	100 mL
PA-1503	OsteoImage™ Mineralization Assay	500 tests

VIII. Product warranty

CULTURES HAVE A FINITE LIFESPAN *IN VITRO*. Lonza guarantees cell performance only when the approved media and supplements are used.

IX. Quality control

Osteoblastic viability of the cryopreserved osteoblasts is guaranteed based upon mineralization assay (alizarin red, a biochemical assay to determine the presence of calcium deposition by cells) and staining for alkaline phosphatase (a biochemical and histochemical marker for primary osteoblasts in proliferative phase). The osteoblasts are guaranteed for at least 12 population doublings and the cells test negative for mycoplasma and bacteria.