MSC Osteogenic Differentiation

This document describes the expansion, osteogenic differentiation and staining of primary human mesenchymal stem cells (MSCs) grown in a CELLnTEC MSC expansion medium.

The following reagents are required for differentiation and staining:

- CnT-Prime MSC Proliferation medium (e.g. CnT-PR-MSC or CnT-PR-MSC-XF)
- CnT-MSCDIFF-OST.S: Osteogenic differentiation supplement (1 mL)
- Mesenchymal stem cells, adipose derived or bone marrow derived
- 10% formalin
- PBS (Ca^{2+} and Mg^{2+} Free)
- Distilled water

Alkaline phosphatase staining:

- Sigmafast BCEP/NBT substrate solution (Sigma product #B5655, one tablet in 10 mL distilled water. Use within 2 hours of preparation)
- Wash buffer (PBS + 0.05% TWEEN)

Alizarin Red staining:

- PBS, pH 4.1-4.3
- Alizarin Red solution (Sigma Product #A5533, 1 g in 50 ml distilled water, pH 4.1-4.3, sterile filtered)

General Notes

Some key factors for successful expansion and differentiation of MSCs are as follows:

- Reproducible differentiation is routinely obtained up to passage 4.
- The passaging protocol during routine expansion (prior to differentiation) is an important consideration. Please follow the seeding density recommended for the proliferation medium you are using, and ensure cells are passaged before reaching 100% confluency. Acctuase is recommended for detaching cells, because it is much gentler than trypsin, does not require a separate reagent to stop the reaction (simply dilute with medium immediately after detachment), and has a larger safe timing window between effective cell detachment and the point where cells are over-digested and damaged. Accutase is heat senstive, and must be stored at 4°C. It does not have to be pre-warmed to detach cells. Exposure to higher temperatures or repeated warming / cooling cycles may lead to reduced activity.
- During fixation and staining, ensure that the cells are not allowed to dry out at any point.

Thawing and Cell Expansion

1. Prepare medium: add the appropriate amount of the desired CnT MSC expansion medium to the culture flasks in which the MSCs will be expanded. Allow medium to equilibrate pH and temperature for at least 30 min at 37°C in the 5% CO_2 incubator.

2. Thaw vial of MSC with gentle swirling in water bath at 37°C until just melted. Vials must be thawed rapidly, whilst ensuring that the temperature of the cells inside does not rise above 4°C (remove vial from water bath whilst a couple of last ice crystals remain). For more explanation about the features of an optimal thawing procedure, please review the knowledge base article in the resources section of www.cellntec.com.
3. Resuspend the cells in the cryo vial by pipetting up and down two to three times and transfer them to the equilibrated cell culture vessels. The DMSO content must be diluted to less than 1%.
4. The volume of cell suspension seeded per vessel should deliver at least the recommended seeding density for the proliferation medium selected (2'000 cells per cm^2). Complete a medium change 6 to 24 h after seeding to remove the residual DMSO from the freezing medium.
5. Expand the cells for the desired number of passages at 37°C and 5% CO_2 with 3 medium changes per week (Mon/Wed/Fri). For best expansion and differentiation, cultures should be maintained without antibiotics / antimycotics. Passage the cells prior to reaching full confluency using Accutase (#CnT-Accutase-100) according to the general cultivation protocol available at www.cellntec.com/products/resources/protocols.

Differentiation

1. After the desired number of expansion passages, seed the cells to be differentiated at approximately 5'000 cells per cm^2 in proliferation medium.
2. The next day, change medium to the desired proliferation medium supplemented with the osteogenic differentiation supplement (#CnT-MSCDIFF-OST.S; 1 ml per 50 ml of medium).
3. Continue to culture the cells in the differentiation medium for 14-21 days, with medium changes 3x per week.

Alkaline Phosphatase Staining

1. After the differentiation period, remove medium, wash the cells with PBS, and fix with 10% formalin solution for 60 seconds at room temperature (not longer, to avoid enzyme deactivation)
2. Remove formalin and wash with wash buffer. Add BCIP/NBT substrate solution (250 uL per cm^2) and incubate until the desired level of staining is achieved (typically approximately 6 minutes, check every 2 minutes).
3. Remove substrate solution, and wash with washing buffer.
4. Add distilled water (250 uL per cm^2), and the cells are ready for imaging. Undifferentiated cells will show slight blue color, with the colour intensity increasing with differentiation.

Alizarin Red staining

1. After the differentiation period, remove medium, wash the cells with PBS pH 4.1-4.3, and fix with 10% formalin solution for 30 minutes at room temperature.

2. Remove formalin and rinse with distilled water.
3. Add Alizarin Red solution (250 uL per cm^2) and incubate in the dark for 45 min at RT.
4. Remove Alizarin Red solution and wash twice with distilled water.
5. Add PBS pH 4.1-4.3 (250 uL per cm^2) and the cells are ready for imaging. Undifferentiated cells (without calcium deposits) stain slightly reddish, differentiated osteoblasts appear bright orange red with calcium deposits.

Further questions? CELLnTEC scientists work regularly with these cultures, and are available in case further questions arise. To contact them directly, please email scientist@cellntec.com