***Disclaimers: This protocol is written for the use of the human Epidermis Dissociation kit (Miltenyi Biotec); These protocols were taken from the manufacturers site and adapted for readability and specificity to our experiment

Purpose

To isolate keratinocytes from epidermal skin biopsy samples for further cultivation. Specifically, to do so from AD and PN patients for further study of the diseases in later experiments.

Protocol

Prepare Materials:

1. Prepare Enzyme G:
    a. reconstituted the Enzyme G lyophilized powder in the vial with 3 mL of sterile, distilled water.
        i. Do not vortex.
    b. Produce aliquots of appropriate volume.
        i. Store aliquots at –20 °C. Avoid repeated freeze-thaw-cycles.
2. Prepare aliquots of appropriate volume of Enzyme P
    a. Store aliquots at –20 °C. Avoid repeated freeze-thaw-cycles.
3. Prepare Enzyme A:
    a. reconstitute the Enzyme A lyophilized powder in the vial with 1 mL RPMI 1640.
        i. Do not vortex.
    b. Produce aliquots of appropriate volume.
        i. Store aliquots at –20 °C. Avoid repeated freeze-thaw-cycles.
4. Prepare PB Buffer:
    a. Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA) by diluting MACS BSA Stock Solution (# 130-091-376) 1:20 with PBS.
        i. Keep buffer cold (2–8 °C).

NOTE: These reagents are from a single kit and valid for 100 4mm diameter biopsy samples

Separation of epidermis from dermis (Day 1):

1. Wash human skin tissue in an appropriate buffer or cell culture medium, e.g., MACS Tissue Storage Solution.
2. Remove subcutaneous fat using scissors.
    a. <u>If the diameter of the skin sample exceeds 4 mm in diameter, take one or more 4 mm diameter punches by rotating down the tool (e.g., Biopsy Punch) through epidermis and dermis.</u>
        i. Store punches in an appropriate buffer or cell culture medium, e.g., MACS Tissue Storage Solution, until needed.

1. **ALL CELL CULTURE MEDIUMS MUST BE PREPARED IN STERILE ENVIRONMENT - Make sure to label whether AD or PN sample

3. Repeat the following for every 5 samples of skin tissue until you are out of skin sample ***ENSURE all skin tissue samples within the same tube are from the same patient type (i.e. AN or PN):
    a. Transfer 1 mL of RPMI 1640 and 25 µL of Enzyme G into a 2 mL tube and mix carefully.
        i. Keep on ice.
    b. Transfer up to five samples of skin tissue (4 mm diameter) into the 2 mL tube.
    c. Ensure you properly labeled every tube
4. Incubate all samples for 14–18 hours at 4 °C using the MACSmix Tube Rotator (12 rpm).

Automated dissociation of epidermis using the gentleMACSTM Dissociator (day 2):

1. Prewarm the water bath to 37 °C.
2. For each biopsy sample:
    a. take biopsy out of the 2 mL tube and peel off the epidermis from the dermis using curved tweezers.
    b. Prepare enzyme mix:
        i. add 1 mL of RPMI 1640, 25 µL of Enzyme P, and 5 µL of Enzyme A into a new 2 mL tube. Keep on ice. Ensure to label PN or AD properly
            1. ▲ Note: Do not mix Enzyme P and Enzyme A directly.
    c. Transfer epidermis sample into the corresponding 2mL tube containing the enzyme mix.
3. Incubate all samples for 60 minutes at 37 °C in a water bath.
    a. It has to be ensured that the sample material is located in the enzyme mix during the incubation time.
4. For each sample:
    a. Lay the epidermis directly on the rotator/stator of the gentleMACSTM C Tube.
        i. Transfer the remaining liquid of the 2 mL tube in the C Tube.
            1. ▲ Note: It has to be ensured that the sample material is located in the enzyme mix in the area of the rotor/stator <u>before</u> starting the gentleMACS Program as the epidermis might easily stick to the tube wall and won´t be dissociated.
    b. Stop the enzymatic reaction by adding 1 mL of cold PB buffer.
    c. Tightly close C Tube and attach it upside down onto the sleeve of the gentleMACS Dissociator.
        i. ▲ Note: Close C Tube tightly beyond the first resistance.
5. Run the gentleMACS Program B.
6. For each sample:
    a. After termination of the program, detach C Tube from the gentleMACS Dissociator.

b. Perform a short centrifugation step to collect the sample material at the tube bottom.
c. Resuspend the sample by pipetting up and down and apply the cell suspension to a Pre-Separation Filter, 70 µm placed on a 15 mL tube.
    i. ▲ Note: Dissociated tissue can be removed from the closed C Tube by pipetting through the septum-sealed opening in the center of the cap of the C Tube. Use ART 1000 REACH 1000 µL pipette tips.
d. Wash the Pre-Separation Filter, 70 µm, with 1 mL of cold PB Buffer.
    i. ▲ Note: (Optional) To collect remaining cells in the C Tube add buffer first to the C Tube and then on top of the filter.
e. Discard the Pre-Separation Filter, 70µm, and centrifuge sample at 300×g for 10 minutes at room temperature. Aspirate supernatant completely
f. Resuspend cells by pipetting up and down with PB buffer or an appropriate buffer to the required volume for further applications.
    i. ▲ Note: If cell clumps occur after the washing step, add another 10 µL of Enzyme A per mL of cell suspension, mix gently, incubate for 5 minutes at 37 °C in a water bath, and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely and repeat step 16.
g. Process cells immediately for further applications.
    i. Typical target cell yields per 4 mm punch biopsy of adult abdominal skin are about 8×10^4 total cells (mostly keratinocytes) and 3×10^3 Langerhans cells.