DETAILED PROTOCOL

NCR minute biopsy • TIMING 20 min for each tissue specimen

1. Take the small tissue NCR (10-20 mm^3) specimen from normal colorectal mucosa after surgery excision and transfer it to a 50 ml conical tube containing 10 ml of cool (+4°C) NCR harvesting medium.
2. Under a sterile biosafety laminar flow hood, remove the NCR harvesting medium (as described in "Reagent and equipment details" supplemental information) and rinse the tissue specimen with 5 ml of sterile NCR wash solution (as described in "Reagent and equipment details" supplemental information). Pipette gently up and down 3-4 times and discard the solution. Repeat the step for three times.
3. Lift up the tissue specimen with a disposable sterile plastic tweezer and place it in a 100 mm cell culture dish. Using the tweezer and a sterile disposable scalpel finely cut the tissue specimen into small 2-3 mm^3 pieces.
4. Place 4 ml of DMEM F12 medium serum free into the cell culture dish, aspirate the minced NCR specimen and transfer all the pieces into a T25 flask. Prepare one T25 flask for each NCR specimen.
5. Add 500 µl of 0.7% Type II collagenase enzyme solution in each T25 flask.
6. Incubate the T25 flask overnight at 37°C, with 5% CO_2.

NCR tissue culture set up • TIMING 20 min

7. The following day, transfer the entire content of the T25 flask into a 15 ml tube and centrifuge at 200 g at RT, for 5 min.
8. Discard the supernatant and wash the cell pellet with 2 ml of sterile NCR wash solution (as described in "Reagent and equipment details" supplemental information) by gently pipetting up and down and centrifuge at 200 g at RT, for further 5 min.
9. Resuspend the cells in 2 ml of complete DMEM F12 and mix gently.
10. Count 10 µl of cells by diluting 1:1 in Trypan blue and using the Burker's chamber. Approximately $1x10^4$-$2x10^5$ cells will be obtained from the NCR tissue specimen digestion.
11. Transfer the NCR cell suspension into a new T25 flask (T0) and add further 2 ml of complete DMEM F12.
12. Incubate at 37 °C, with 5% CO_2 overnight.

NCR tissue culture cell recovery • TIMING cell recovery and medium changes: 7 min plus 7 days culture

13. The following day, before feeding the T0 flask with new medium, collect any loosely attached or floating rounded cells into a 15 ml conical tube and centrifuge at 200 g at RT, for 5 min.
14. Resuspend the cell pellet in 4 ml of complete DMEM F12, mix gently and reseed cells in a 6-well plate (T1). Incubate T0 flask and T1 6-well plate at 37°C and 5% CO_2 for 2 days.
15. After two days, wash the attached cells in T25 flask (T0) and 6-well plate (T1) with DPBS 1X and replace them with fresh complete media.
16. Incubate at 37°C, with 5% CO_2. Leave cells to grow for additional 5 days, changing the medium twice weekly. During this period, check the formation and development of the cell colonies on the inverted microscope, daily.

NCR keratinocyte culture enrichment, isolation and characterization • TIMING keratinocyte enrichment: 7 days culture; keratinocyte isolation: 30 min for five colonies; characterization: 4 h

17. After 7 days in complete DMEM F12, start adding 50% of dKSFM to the NCR derived cell culture (T0) (T1) for further 7 days. Leave primary cell cultures to grow, changing the medium twice weekly. During this period, check colony development on the inverted microscope, daily. At this point, it is useful to draw small circles around the colonies (outside the bottom part of the 6-well plate (T1)), in order to identify the keratinocyte colonies.
18. After 14 days of culture, analyse the colonies on the inverted microscope and select the larger and purer ones from the T1 culture.
19. Remove the DMEM F12/dKSFM (1:1 ratio) medium from T1 and wash cells twice with DPBS 1X. Completely remove the DPBS.
20. Following the circles drawn outside the 6-well plate, place a cloning ring around each colony and seal it with a silicone rubber laboratory pressing lightly down so that the bottom of the cloning ring can adhere well to the 6-well plate.
21. Add 100 µl of Trypsin/EDTA solution in each cloning ring and incubate at 37°C for 5 min to detach keratinocyte colony.
22. Inactivate trypsin activity by adding 400 µl of complete DMEM F12 medium in each cloning ring. Mix the cells well by pipetting up and down.
23. Collect the cells in a 15 ml tube and centrifuge at 200 g at RT, for 5 min.

24. Resuspend the cells in 2 ml of 100% dKSFM and seed each colonies in a single well of a new 6-well plate (T2).
25. At the same time, cell cultures T0 from Step 17, are characterized for the expression of cytokeratins by immunofluorescence assay (see Steps 28-40).

NCR keratinocyte culture maintenance, expansion and characterization • TIMING maintenance and expansion: up to confluence; characterization: 4 h

26. Incubate the T2 cultures in presence of 100% dKSFM at 37°C, with 5% CO_2. Change the dKSFM medium twice weekly. Wait for the cell culture to become confluent, which typically takes 2-3 weeks.
27. As in Step 25, verify the phenotype of T2 cultures maintained in 100% dKSFM medium by immunofluorescence assay (see Steps 28-40). Expanded cell cultures can grow for up to confluence.

NCR keratinocyte culture characterization by immunofluorescence assay • TIMING 4 h

28. Seed NCR primary cells ($5x10^3$ cells) on cover glasses in presence of DMEM F12/dKSFM (1:1 ratio) medium (Step 17 and 25) or 100% dKSFM medium (Step 27). Incubate the cells at 37°C, with 5% CO_2.
29. After 2 days, discard the media and wash the cells with DPBS 1X for 3 min (3 times).
30. Fix the cells with 10% of neutral buffered formalin for 7 minutes at RT.
31. Wash the cells with DPBS 1X for 3 min (3 times).
32. Permeabilize cell with 300 µl of the cell permeabilization solution for 2 min.
33. Block using 300 µl the blocking solution for 30 min RT.
34. Stain the cells with 50 µl of primary antibody solution for 2 h, at RT.
35. Wash the cells with PBS 1X for 3 min (3 times).
36. Incubate the cells with 50 µl of secondary antibody solution for 1 h, at RT in the dark.
37. Wash the cells with DPBS 1X for 3 min (3 times).
38. Counterstain nuclei with 50 µl DAPI.
39. Seal the edges of each cover glasses onto a microscope slide with regular transparent nail polish and allow to dry in the air for 3 min.
40. Acquire images under the fluorescence microscope.