Standard operating procedure for primary bovine mammary epithelial cell (pbMEC) isolation

Leibniz Institute for Farm Animal Biology (FBN)

Institute of Genome Biology, Genome Physiology Unit

Written by Doreen Becker (becker.doreen@fbn-dummerstorf.de)

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Purpose

Collecting pbMECs directly from the cow's udder for cell isolation. Appropriate preparation of required materials and professional training of lab technicians involved in pbMEC cell preparation have to be performed in advance.

Materials

- large cutting knife
- H_2O, 70% EtOH, 1X HBSS buffered with 0.2% 1M HEPES
- antibiotic-antimycotic solution
- biosafety cabinet
- collagenase
- water bath
- mesh steel grid gradient
- 50ml tubes
- centrifuge
- FBS, DMSO
- cryovials, liquid nitrogen
- cell culture medium*, RPMI1640-medium
- collagen-coated cell culture plate, non-coated cell culture plate
- cell culture incubator
- 1X PBS, Trypsin-EDTA solution
- microscope, cell scraper
- Cellometer Auto2000

Hazard warning:

- Liquid nitrogen is a hazardous chemical. It is nontoxic but cryogenic, asphyxiant gas that can reduce the normal oxygen concentration in breathing air. Exposure to oxygen-deficient atmosphere may cause dizziness, drowsiness, nausea, diminished mental alertness, loss of consciousness and suffocation. Contact of liquid nitrogen with skin and eyes can cause severe cryogenic burns.
- Anyone handling cryogenic liquids and asphyxiant gas should be familiar with the hazards presented by such materials and trained in how to handle them safely. Liquid nitrogen should be used in a well ventilated area. To prevent personal hazards due to cryogenic liquids users have to wear personal protective equipment.

pbMECs isolation

1. the animals are killed according to regular slaughtering protocol including stunning and exsanguination by expert staff in an experimental slaughterhouse of the institute

2. the udder is cut off immediately after slaughter by expert staff and distributed to trained lab technicians for further sample preparation

3. the skin of the udder is cleaned with H_2O and rinsed with 70% EtOH

4. a large cut using a clean knife is made to open the gland sinus and the lactiferous ducts draining into the gland sinus

5. fist-sized tissue pieces are cut out of the udder tissue and immersed into 4°C cold 1X HBSS solution buffered with 0.2% 1M HEPES

6. each tissue piece is rinsed twice with 1X HBSS solution buffered with 0.2 % 1M HEPES

7. tissue piece is immersed in 1X HBSS solution buffered with 0.2 % 1M HEPES and containing 2% antibiotic-antimycotic solution (Penicillin 10.000U/ml / Streptomycin 10mg/ml) and kept at 4°C (protocol is continued directly or the next day under a biosafety cabinet)

8. fist-size tissue pieces are rinsed with 1X HBSS solution buffered with 0.2 % 1M HEPES

9. tissue pieces are cut into slices as thick as a finger and slices are cut into strips (~5mm)

10. tissue pieces are immersed into 1X HBSS solution buffered with 0.2 % 1M HEPES and repeatedly shaken before large clumps are allowed to settle until the supernatant remained clear of visible clumps

11. supernatant is removed and step 10 repeated

12. supernatant is removed

13. tissue pieces are immersed into 1X HBSS solution buffered with 0.2 % 1M HEPES and supplemented with 200 U/ml collagenase, type IV and shaken at 37°C for 45 min and the supernatant is removed

14. tissue pieces are immersed into 1X HBSS solution buffered with 0.2 % 1M HEPES and supplemented with 200 U/ml collagenase, type IV and shaken at 37°C for 45 min and the supernatant is filtered through a mesh steel grid gradient (500 µm, 300 µm, 150 µm and 90 µm) and collected

15. step 14 is repeated twice (continue with step 16 to step 24 during the digestion step (step 14))

16. filtrate is diluted with 1X HBSS solution buffered with 0.2 % 1M HEPES to thin down collagenase concentration (at least 2:3 dilution)

17. diluted filtrate is dispersed into 50ml tubes and centrifuged at 1000 rpm for 10 min (brake set to off)

18 supernatant is removed and the pellet is resuspended with 1 ml 1X HBSS solution buffered with 0.2 % 1M HEPES

19. all resuspended pellets are pooled into one 50 ml tube

20. tube is centrifuged at 1000 rpm for 10 min (brake set to off) and the supernatant is removed

21. pellet is resuspended with 1 ml 1X HBSS solution buffered with 0.2 % 1M HEPES before 49 ml of 1X HBSS solution buffered with 0.2 % 1M HEPES are added

22. tube is centrifuged at 1000 rpm for 10 min (brake set to off) and the supernatant is removed

23. step 21 and 22 are repeated 5 times

24. pellet is resuspended with 1 ml FBS (fetal bovine serum) and kept at 4°C

25. 1ml freezing medium (80% FBS, 20% DMSO) is added drop by drop

26. 1ml of the cell suspension are transferred to a cryovial

27. cryovial is kept at -80°C for at least 24 h up to one week before it is stored in liquid nitrogen

- The experimental procedures were carried out according to the animal care guidelines with respect to welfare and health of the animals and were approved by the relevant authorities of the State Department of Agriculture, Food Security and Fisheries, Mecklenburg-Western Pomerania, Germany.

pbMECs cultivation

1. cryovial is thawed at 37°C

2. cell suspension is transferred to 10 ml cell culture medium*

3. suspension is centrifuged at 1000 rpm for 5 minutes at 15°C

4. 10ml cell culture medium is added to a 10 cm collagen-coated plate and supplemented with 100 µl Penicillin-Streptomycin (10.000U/ml / 10mg/ml).

5. cell pellet is resuspended in 1 ml cell culture medium

6. 250 µl of the cell suspension are added to the collagen-coated plate

7. plate is softly shaken to distribute the cell suspension

8. plate is incubated at 37°C with an atmosphere of 5% CO2

9. every other day the medium is discarded and the cells are washed twice with 10 ml 1X PBS

pbMECs harvesting

1. after 6 days of unperturbed growth, medium is discarded and the cells are washed twice with 10 ml 1X PBS

2. 1ml of Trypsin-EDTA solution (0.25% Trypsin /0.02% EDTA in 1X PBS) is added and plate is incubated at 37°C

3. after 5 min 5 ml 1X PBS is added to the detached cells (mainly fibroblasts) and the cell suspension is removed and discarded

4. 1ml of Trypsin-EDTA solution is added to the plate and plate is incubated at 37°C

5. the process of cell detachment is controlled by microscopy

6. after 5 min or when most cells detached from the plate, 5ml cell culture medium is added and cells are softly scraped from the plate using a cell scraper

7. cell suspension is transferred to a falcon tube

8. 5ml of cell culture medium is added to the plate and the fluid is added to the falcon tube of step 7

9. the tube is centrifuged at 1000 rpm for 5 min at 15°C

10. supernatant is removed and pellet is resuspended using 1ml cell culture medium

11. 9ml cell culture medium is added and the cells are plated on a non-coated cell culture plate

12. plate is incubated at 37°C for 30 min

13. cell suspension is carefully collected in a falcon tube

14. tube is centrifuged at 1000 rpm for 5 min at 15°C

15. supernatant is removed and the pellet is resuspended in 1ml pure RPMI1640-medium (very low endotoxin content)

16. Cell concentration and viability are measured using a Cellometer Auto2000 (Nexcelom)

- *cell culture medium:
  to obtain 500 ml of medium combine 439.25 ml RPMI1640 medium (very low endotoxin content), 50 ml of FBS and 10 ml of 200 mM L-Glutamine. Add 250 µl of 200 mM L-Methionine, 200 µl of 1M L-Lysine, 100 µl of Prolactin (5mg/ml), 100 µl Insuline (5mg/ml), 50 µl of 100 mM Sodium pyruvate and 50 µl of Hydrocortisone (10mg/ml)