WELLCOME SANGER INSTITUTE

STANDARD OPERATING PROCEDURE PACKET

Ear epidermal sheet preparation

Isolation and fixation of ear epidermis

Reagents & Buffers

- PBS 1x pH 7.4 without Ca^{2+} and Mg^{2+} (Gibco, Cat no. 10010-015)
- Hair removal cream (Nair)
- 0.5 M ammonium thiocyanate (Sigma Aldrich, Cat no. A7149-100G) in PBS, stored at room temperature and protected from the light, prepared fresh every week.
    - How to prepare:
        - Add 1.9 g of ammonium thiocyanate into 50 ml PBS 1x pH 7.4
        - Make 6 ml for each pair of mouse ears
- PBS 1x pH 7.4 with 0.05% Sodium Azide, stored at room temperature
- Ice cold acetone, stored at -20˚ C

Materials

- 50 ml Falcon tubes and 1.7 ml eppendorfs (Axygen, Cat no. MCT-175-C-PK)
- Forceps from FST
- Petri dishes 50 mm (round) and 100 mm (square)
- 12 and 24-well plates with flat bottom
- Kimwipes
- Waste containers for PBS, ammonium thiocyanate and acetone

Equipment

- Incubator (Heratherm model from ThermoScientific)

Ears are collected and stored in 1.7 ml eppendorfs containing PBS 1x pH 7.4 at room temperature. Samples should be processed on the same day.

1. In advance set the incubator at 37˚ C and prepare:
    - 12-well plates with 1.5 ml of 0.5 M ammonium thiocyanate per well. For each mouse, 4 wells are needed. Place the plates on ice, protected from light.
    - 24-well plates with 1 ml of PBS 1x pH 7.4 per well, 2 wells per mouse. Place the plate at room temperature.
    - 10 cm petri dish with rows of 2 blobs of Nair hair removal cream; 1 blob for each ear.
    - 50 ml falcons filled with 35-40 ml of PBS 1x pH 7.4. Write “PBS1” and “PBS2” on the lids. At the bench place a timer, a set of tools, kimwipes and a 10 cm petri dish facing down.
2. Assign a mouse for each row of Nair and write down its ID. Also register down mouse ID number in all plates and 1.7 ml eppendorfs for upcoming fixation.
3. Dry ears on kimwipes and cover them with Nair hair removal cream, one ear in each blob.
4. Incubate each set of ears for 4 min at room temperature.
5. Gently wipe off most of the Nair using a kimwipe.
6. Transfer both mouse ears into a 50 ml tube filled with PBS (PBS1 tube) and shake vigorously to wash off remaining cream.
7. Transfer into a new 50 ml tube filled with PBS (PBS2 tube) and shake again. Can reuse PBS1 and PBS2 tubes for several ears.
8. Dry ears on kimwipes. On a 10 cm petri dish lid, split each ear into ventral and dorsal halves using 2 forceps. Start at the part of the ear that was cut and work to the edge.
9. Place ear halves in the previously prepared plate containing 0.5 M ammonium thiocyanate with the inner/dermal side facing down - this side appears wet compared to the outside and will float on the ammonium thiocyanate. Use one well for each half and 4 halves in a single row of the 12-well plate.
10. Incubate each plate for 35 min at 37° C.
11. Remove the plate from the incubator and place the plate on ice to stop the process.

12. Transfer all 4 ear halves from one mouse into a 100 mm petri dish with PBS 1x pH 7.4. Separate the epidermis - floating and transparent sheet - from the dermis, using choice of fine forceps as preferred.
13. Place the epidermal sheets in the 24 well plate with 1 ml PBS 1x pH 7.4 per well – 2 sheets per well. Dispose of the dermis in a separate bag.
14. Prepare 1.7 ml eppendorfs filled with 1 ml cold acetone, and leave them on ice.
15. Transfer all 4 epidermal sheets of each mouse into the designated eppendorf.
16. Incubate for 20 min on ice to fix and permeabilize the sheets.
17. Remove the acetone with a filter tip, add 850 µl PBS 1x pH 7.4 to wash. Suck off the PBS and add 1.7 ml PBS with 0.05% Sodium Azide (2 x 850 µl).
18. Store at 4° C until further use.

Epidermis immunophenotyping

Protocol for staining of epidermal sheets and preparation of slides

Reagents & Buffers

1. Staining buffer (PBS (-Mg/-Ca), 2% FCS)
2. PBS
3. Anti-mouse Vγ3 TCR FITC (clone 536, BD 553229), use 1:500
4. Anti-mouse I-A/I-E-A647 (clone M5/114.15.2, Biolegend 107618), use 1:500
5. Anti-mouse CD45 eFluor450 (clone 30-F11, eBioscience 48-0451-82), use 1:200
6. Prolong Gold solution (New Enlgand Biolabs 9071S), defrost before use
7. Nail polish

Materials

1. 1.7 ml microfuge tubes
2. Forceps (Dumont #7)
3. Frosted slides (VWR 631-0108)
4. Coverslips (VWR 631-1379)

Equipment

1. Table top shaker
2. Stereomicroscope

Samples are shipped as fixed epidermal sheets in 1.7 ml tubes containing PBS / 0.5 mM sodiumazide on ice from WTSI to KCL (approximately 2 hours by courier) and processed the same week (samples can be kept in this solution for a few months if restaining is required).

1. Prepare one 1.7 ml tube with 180 µl staining buffer per sample.
2. Put a large drop of staining buffer on a slide. Under the stereomicroscope, place an epidermal sheet on top and unfold the sheet using forceps.
3. Transfer the epidermal sheet into the prepared 1.7 ml tube, making sure it does not fold up again and is exposed to the solution. Use a pipette tip to unroll the sheet again if necessary.
4. Incubate at room temperature for 60 minutes in order to block unspecific antibody binding sites.
5. Prepare the antibody master mix: per sample use 18.2 µl staining buffer, 0.4 µl anti-Vγ3 FITC, 0.4 µl anti-MHC II AF647 and 1 µl anti-CD45 eFluor450.
6. Add 20 µl antibody mix to each tube and incubate for 75 minutes at 37°C and 450 rpm on a table top shaker.
7. Wash samples 4 times for 3-5 minutes with 0.5 ml PBS. Use a pipette to remove and add liquid. Store in the fridge until slide preparation.
8. To prepare slides set up a stereomicroscope with a table top light and fibre optics illumination. Using a stereomicroscope helps to identify the dermal side of the epidermal sheet and stretch the epidermal sheet on the slide efficiently (hair side down, if no hair is present this side looks rougher).
9. Transfer the epidermal sheet onto a labelled slide and use forceps to stretch it out dermal side down
10. Place a drop of Prolong Gold solution on the epidermal sheet and cover with a coverslip. Gently press down the coverslip with the forceps, squeezing out any bubbles.
11. Seal the coverslip with nail polish. The slide is now ready to be imaged.