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Cell culture

1. Cell culture media

**Supplements**

* Hydrocortisone/EGF (1000X): dissolve hydrocortisone (Sigma H-0888) in 100% ethanol at 0.5 mg/ml. Mix 1 ml of this with 19 ml DMEM containing 2.5 µg EGF (Invitrogen PHG0314 or DBio DBEG-EGF-H1). Store sterile 550ul aliquots at -20°C (HC = 25 µg/ml; EGF = 0.125 µg/ml).
* Insulin (5 mg/ml): dissolve 100 mg (Sigma I-5500 or I-2643) in 20 ml distilled water containing 200 µL glacial acetic acid. Filter and store 550ul aliquots at -20°C.
* Cholera toxin (11.7 µM): dissolve 1 mg vial (Sigma C-8052) in 1 ml distilled water and filter sterilize. Stable at 4°C for about 1 year.
* Gentamicin (10 mg/ml): Gibco 15710-049. Store at 4°C.
* Amphotericin B (250 µg/ml): Fisher 10746254. Sterile 550 µl aliquots stored at -20°C.
* Y-27632: dissolve 25 mg (Cambridge Bioscience Y1000-25mg) with distilled water to stock concentration 5 mM (15.61 ml dH₂O), filter, make 550 ul aliquots and store at -20°C.

**DMEM**

Complete DMEM for J2s: DMEM high glucose w/Pyr+Gln + 50 mL **adult** bovine serum + 5 mL pen/strep

**FMED**

A: DMEM high glucose w/Pyr+Gln + 50 mL **fetal** bovine serum + 5 mL pen/strep + 500 μL amphotericin B (250 μg/mL stock) + 500 μL of gentamicin (10 mg/mL stock)

B: Ham’s F12 + 5 mL pen/strep + 500 μL amphotericin B + 500 μL of gentamicin (10 mg/mL stock)

1. Remove 182 mL from A, store in 100 mL pots for reuse.

2. Add 125 mL of B, store bottle for future use.

3. Add supplements:

- 500 μL of Rock inhibitor Y-27632 (5 mM stock)

- 500 μL of insulin (5 mg/mL stock)

- 500 μL of hydrocortisone (25 µg/mL stock) + EGF (0.125 µg/mL stock)

- 4.3 μL cholera toxin (11.7 µM = 1 mg/mL stock)

**Complete FAD**

50% DMEM + 50% DMEM/F-12 + 25 mL FBS + 5 mL pen/strep + 500 μL amphotericin B (250 μg/mL stock)

- 500 μL of apo-transferrin (5 μg/ml final; 5 mg/mL stock)

- 500 μL of insulin (5 μg/ml final; 5 mg/mL stock in 1% acetic acid)

- 500 μL of hydrocortisone (500 ng/mL final) + EGF (10 ng/mL final)

- 4.3 μL cholera toxin (0.1 nM final; 1 mg/mL stock)

- 0.18 mM adenine final (add 12.15 mg in 5 mL water, microwave for 3 s)

**Minimal FAD**

50% DMEM + 50% DMEM/F-12 + 25 mL FBS + 5 mL pen/strep + 500 μL amphotericin B (250 μg/mL stock)

- Apo-transferrin (5 μg/ml final)

- 500 μL of insulin (5 mg/mL stock)

**Sphere medium**

DMEM/F-12 with glutamax + 5 mL pen/strep + 500 μL amphotericin B (250 μg/mL stock)

- B27 (2% of 50x, full bottle in 500 mL)

- FGF2 (20 ng/mL final; stock 100 μg/ml)

- FGF7 (20 ng/mL final; stock 10 μg/ml)

**BEGM**

BEBM Basal Medium ([**CC-3171**](https://bioscience.lonza.com/p/000000000000185310)) + BEGM SingleQuots Supplement Pack ([**CC-4175**](https://bioscience.lonza.com/p/000000000000185383)):

BEGM:  
- Orange Cap Vial with BPE, 2.00 mL  
- Lilac Cap Vial with Insulin, 0.50 mL  
- Natural Cap Vial with Hydrocortisone, 0.50 mL  
- Red Cap Vial with GA-1000, 0.50 mL  
- Amber Vial with Retinoic Acid, 0.50 mL  
- Natural Cap Vial with Transferrin, 0.50 mL  
- Amber Vial with Triiodothyronine, 0.50 mL  
- Amber Vial with Epinephrine, 0.50 mL  
- Green Cap Vial with hEGF, 0.50 mL

**Keratinocyte SFM**

Keratinocyte-SFM Basal Medium + 5 mL pen/strep + BPE + EGF

- 25 mg Bovine Pituitary Extract (BPE) stock  
- 2.5 μg EGF stock

1. Cell culture

**Thawing cells**

1. Add 5 mL of medium to T25 (or 12 mL to T75).
2. Keep cryovials in dry ice and then quickly thaw in 37 °C water bath.
3. Pipette cells into flask and keep in suitable incubator (7.5% CO2 for DMEM, 5% CO2 for FMED and RPMI)

**Preparing feeders**

1. Wait until 3T3-J2 fibroblasts in T75 are 80% confluent.
2. Decant or aspirate medium and add 150 μL of 4 μg/mL mitomycin in 7 mL for 3-4 h.
3. Split and count cells, seeding 300,000 to 500,000 cells per T25.

**Airway explanting** (for pure epithelial and fibroblast cultures see ***Magnetic separation***)

1. Keep airway resection or biopsy at 4 °C in transport media until ready.
2. Wash airway with PBS in Petri dish to remove blood.
3. Cut away parenchyma and section into 3x3 mm fragments with tweezer and scalpel.
4. Place fragments with luminal side down on feeder flask and cover with 1.5 mL FMED.
5. Progressively increase medium to 5 mL by changing medium every 2/3 days.

**Splitting, seeding and freezing cells**

1. Decant or aspirate medium and wash flask with PBS.
2. Add 1 mL of trypsin/EDTA to T25 (or 2 mL to T75).
3. If removing feeders, after 2 min, repeat 1 & 2 making sure that no fibroblasts remain.
4. After 5 min, tap flask, add 2 or 3 mL of medium and mix well by pipetting to dislodge and dissociate cells.
5. Pipette into 15 mL Falcon and spin down at 300 g for 5 min if necessary.
6. Pipette desired **split** into new flasks with 5 mL of medium per T25 (12 mL per T75).
7. For **seeding**, count 10 μL in haemocytometer.
8. Seed cells according to experiment.

- For standard 7 day experiments, seed 200,000 cells/well in 6-well plates, 100,000 cells/well in 12-well plates or 40,000 in 24-well plates.

- For IC50 screens, seed 2000 cells/well in 96-well plates with FMED.

- For high-throughput screens, seed 2000 cells/well in 384-well plates with cFAD.

1. For **freezing**, keep pellets in separate tube.
2. Prepare freezing medium

* For 3T3-Js: complete DMEM with 20% FBS + 10% DMSO
* For HBECs: 50% FMED + 42.5% Lonza Pro-Freeze + 7.5% DMSO
* Alternatively, use premade freezing media:

- Recovery cell freezing medium (= DMEM + 10% FBS + 10% DMSO)

- Crystor 5 and Crystor 10 (serum free)

- Bambanker (serum free, direct to freezer)

- Cellbanker 2 (with FBS, direct to freezer)

1. Pipette 500,000 cells in 300-500 μL into each cryovial.
2. Place in Mr Frosty at -80 °C for at least 3 h.
3. PBMC isolation
4. Check centrifuge is at room temperature. Bloods can be kept at room temperature.
5. **Blood control**: Aspirate 600 μL of whole blood from one EDTA tube and reserve in a separate Eppendorf tube.
6. Transfer 5 mL blood from the EDTA tube to a labelled 50 mL falcon tube and add 10 mL PBS
7. Take two 50 mL falcon tubes and add 15 mL Ficoll to each (Ficoll bottle must be wrapped in tin foil when taken into the light).
8. Carefully layer 15 mL diluted blood into each Falcon tube onto the Ficoll using a 20 mL stripette onto the side of the tube.
9. Spin down tubes at 1200 G with 0 brake and 0 acceleration (this will take about 40 mins) for 30 min at RT.
10. Carefully aspirate the plasma with a sterile disposable Pasteur pipette and transfer into 2x 15 mL falcon tubes.
11. Carefully aspirate the buffy coat (mononuclear cells) and transfer to the other 15 ml falcon tube and top up with PBS.

**Plasma processing**

1. Spin down tubes at 3000 G for 10 min at RT (9 brake and 9 acceleration).
2. Aliquot plasma without touching the pellet into 1.5 mL eppendorfs – aliquot 1-1.5 mL

plasma per tube.

**IMDM preparation**

1. To prepare autologous IMDM, add 5 mL plasma to 45 mL IMDM with 500 μL pen/strep and 50 μL amphotericin B.

**Buffy coat**

1. Spin down at 1200 G for 10 min at RT (9 brake and 9 acceleration).
2. Suction off the supernatant and resuspend the pellet in 5 mL PBS.
3. Spin down at 300 G for 5 min at RT (9 brake and 9 acceleration).

**PBMC freezing**

1. Suction off the supernatant and resuspend the pellet in 5 mL PBS.
2. Count PBMCs and prepare number of aliquots so that 0.5 to 10 millions PBMCs/mL in each.
3. Spin down at 300 G for 5 min at RT (9 brake and 9 acceleration).
4. Remove supernatant with and resuspend the pellet in freezing medium (90% FBS:10% DMSO).
5. Transfer in 1 mL aliquots to individual cryovials and freeze in Mr Frosty.

**Macrophage culture**

1. Suction off the supernatant and resuspend the pellet in 10 mL autologous IMDM.
2. Culture on a T75 for up to 2 weeks so that monocytes can attach and differentiate.
3. Epithelioids

**From tissue**

1. Cut resected bronchi into very small chunks with a scalpel and place luminal side down on Greiner inserts (0.4 μM): 4 chunks per 6-well insert or 1 chunk per 24-well insert.
2. Cover with FMED (or cFAD): 300 μL per 6-well insert or 50 μL per 24-well insert. Outside medium: 2 mL in 6wp and 1 mL in 24wp.
3. Change inside medium after 3 days and top up medium to 1 mL (6wp) or 200 μL (24wp) when cells begin to grow out (7 days) - treat as needed then. Treat same days of week (e.g. Mon, Wed, Fri) and change media when yellow, before treating. If sequencing supernatant, inside medium should only be changed once a week when collecting.
4. Once cells are around 90% confluent (3-4 weeks), remove chunks and place onto new inserts.
5. Once wells are fully confluent (4-5 weeks), change medium to mFAD (inside and outside). Can change medium to 50:50 FMED:mFAD (or 100% mFAD oustide) a few days before.

**From cells**

1. Trypsinise cells and resuspend in 1 mL of FMED in 15 mL Falcons.
2. Add 5 μL DiI or DiD to the different tubes, take 10 μL for counting and place in water bath at 37 °C for 30 min.
3. Count cells during incubation.
4. Spin 300 g for 5 min, wash with PBS, spin again.
5. Combine equal numbers of labelled cells and seed 300k cells per well (so that each well contains 150k DiI- and 150k DiD-labelled cells).
6. Start treatment after 48 h or upon confluence.

**Endpoints**

- **Cell competition**: image cells with epifluorescence/confocal microscope longitudinally; run flow cytometry at endpoint.

- **Clonal diversity**: harvest supernatant from insert every week for Nanoseq; harvest DNA/RNA from tissue at endpoint; tissue chunks can be frozen or reused.

- **Gene expression**: harvest RNA/protein from tissue at endpoint; fix tissue for IF (membranes can be removed and cut for multiple stainings on slides).

1. 3D cell culture in self-assembling PA gels
2. 3D cell culture in collagen gels
3. 3D cell culture in Matrigel
4. Cigarette smoke and vape extract preparation

**Cigarette smoke extract**



1. Prepare 10 mL PBS in 15 mL falcons, one per cigarette. Cover top with parafilm.
2. In a fume hood, set up a piece of aluminium foil large enough to catch all the ash and fold it to hold a cigarette. Place a small beaker with water next to it.
3. Prepare cigarette by removing filter and wrapping a small piece of parafilm around its end. Then fit this end 2.5 mL syringe and connect to luer lock system. Place 50 mL syringe on central lock and extension tubing into falcon, through parafilm into the bottom.
4. Light the cigarette and pull the 50 mL syringe to burn it and draw smoke. It should become completely opaque, with a cream color.
5. Continue to draw smoke through the system until the line is reached on the cigarette – this is usually 7 draws. At this point extinguish the cigarette using water.
6. Cover falcons in foil and filter using a 0.2 μm filter in a tissue culture hood.
7. Store filtered 100% CSE solutions in amber eppendorfs at -80 °C.

**Vape extract**

1. Connect 50 mL syringe to luer tubing and cut it so it fits in the outlet of vape device.
2. Prepare 10 mL PBS in 15 mL falcon. Cover top with parafilm and place extension tubing into falcon through parafilm.
3. Draw vapour into syringe so that it is opaque and release into tube in falcon. Repeat 30 times.
4. Store filtered 100% VE solutions in amber eppendorfs at -80 °C.

RNA & DNA

1. PCR

**Direct PCR**

1. Prepare 20 μL reactions in PCR tube strips or plates: 2 μL sample + 10 μL 2x Taq polymerase master mix + 2 μL 10 μM For + Rev primer mix + 6 μL nuclease-free water.
2. Run samples in thermal cycler for 35 cycles of 30 s at 95°C, 30 s at 55°C, 60 s at 72°C, plus a final extension step of 8 min at 72°C.

**Gel electrophoresis**

1. Make a 1% agarose gel, for a large cassette: 2.5 g agarose in 250 mL 1x TBE.
2. Heat solution in microwave to dissolve agarose, then leave until cool enough to touch.
3. Add 3 μL Gel Red per 100 mL (i.e. 7.5 μL).
4. Add stained agarose to cassette and leave to set.
5. When it is ready, remove plastic comb and fill tank with 1x TBE.
6. Prepare samples by making 1.5 μL drops of loading dye on parafilm.
7. Pipette 4 μL of HyperLadder 50bp into the first well of each row.
8. Mix 9 µL of PCR product each drop of loading dye and pipette 9 μL of the mix into gel.
9. Run gels at 120V (small) or 200V (large) for at least 20 min.
10. qPCR

**RNA extraction**

1. Bucket of ice, all steps to be done on ice.
2. In the fume hood: Lyse cells using **Trizol** reagent, 500 µL per well, pipette up and down until liquid [and: optional freeze at -80°C].
3. Transfer to eppendorfs, add **chloroform**, 1/5 of Trizol vol i.e. 100 µL per well, and shake well.
4. For optimal separation: spin *phase-lock-gel eppendorfs* and transfer mix into them.
5. Spin for 15 min at max speed (>12 000 G).
6. Extract upper phase (transparent), add equal amount of **isopropanol** (200-250 µL per well) with **glycogen** (0.5 µL of 20 µg/µL per 1 mL Trizol, i.e. 0.25 µL) and shake well. (Prepare stock mix first)
7. Leave 10 min at RT [or: optional leave at -20°C overnight].
8. Spin 30 min at max speed, discard supernatant.
9. Add **80% ethanol**, 800 µL, spin 8 min at max speed, discard supernatant.
10. Add **100% ethanol**, 800 µL, spin 8 min at max speed, discard supernatant.
11. Invert and dry at RT (ensure complete solvent evaporation).
12. Add 15 µL **RNAse-free water** and vortex.
13. Measure RNA quality and concentration on Nanodrop.

**cDNA synthesis** (Qiagen kit)

1. Calculate sample volume for 1 µg, add 2 µL gDNAse, top up with RNAse-free water to 14 µL;

if sample volume is over 12 µL, add no water.

1. Wipeout [aka RT1] phase at thermal cycler (2 min at 42°C).
2. Prepare RT mix for all samples +10% (per well: 1 µL RT, 1 µL primer mix, 4 µL RT buffer).
3. Add 6 µL of mix to 14 µL samples and run RT [aka RT2] program on thermal cycler (15-20 min at 42°C, 3 min at 95°C) [and: optional freeze at -20°C].

**qPCR**

1. Dilute 20 µL samples in 180 µL of RNAse-free water for a final concentration of 5 ng/µL. Added volume should be lower for samples <1 µg, adjust accordingly to 5 ng/µL.
2. Prepare mastermix for each gene, with 10% excess volume. Per well: 5 µL SYBR green, 0.2 µL primer mix, 2.8 µL RNAse-free water. Vortex SYBR green/mix before pipetting!
3. Add 2 µL of cDNA samples to 384 well plate (controls: 2 µL water).
4. Spin down plate and add 8 µL of mix to plate.
5. Cover plate with optical film.
6. Insert plate in PCR machine, open software and select program: CT, SYBR green, fast, low ROX, 10 µL volume, default cycles; and input samples and targets for each well.
7. Run samples and save eds file when prompted.
8. After running, check amplification curves and melting curves, and adjust Ct thresholds if necessary.
9. Export sample setup, amplification data and results as xls.
10. Bulk RNA sequencing

**RNA extraction**

1. Wash cells with PBS, trypsinise if necessary and collect into tubes.
2. Add 75-350 μL RLT buffer per sample and vortex.
3. Add each sample to gDNA eliminator column.
4. Centrifuge at 10,000 G for 30 s.
5. Discard *column* and add same volume (350 μL) of 70-80% EtOH to flowthrough.
6. Mix well with pipette and transfer sample (700 μL) to MinElute column.
7. Centrifuge at 10,000 G for 15 s, discard flowthrough and reuse collection tube.
8. Add 700 μL RW1 buffer.
9. Centrifuge at 10,000 G for 15 s, discard flowthrough and reuse collection tube.
10. Add 500 μL RPE buffer.
11. Centrifuge at 10,000 G for 15 s, discard flowthrough and reuse collection tube.
12. Add 500 μL 80% EtOH.
13. Centrifuge at 10,000 G for 2 min, discard flowthrough *and* collection tube.
14. Place column on new 2 mL collection tube.
15. Centrifuge at 20,000 G for 5 min with lid open to dry membrane, discard flowthrough *and* collection tube.
16. Place column on new 1.5 mL collection tube.
17. Add 15 to 50 μL to center of membrane.
18. Centrifuge at 20,000 G for 1 min, keep flowthrough and discard column.

**Qubit RNA quantification**

1. Set up required tubes: n samples + 2 standards.
2. Prepare working solution: 0.2 \* (n+2 +2 extra) mL of RNA HS buffer + 1/200th of RNA HS reagent.
3. Add 199 μL of reagent to sample tubes and 190 μL to standard tubes.
4. Add 1 μL per sample and 10 μL of standard 1 & 2.
5. Vortex for 5 s and stand at RT for 2 min.
6. Run samples in Qubit fluorometer: RNA High Sensitivity, read standard 1, read standard 2, read samples. Lid must be closed during readings. Note down values one by one.
7. Bulk DNA sequencing

**DNA extraction**

1. For cell cultures: wash & trypsinize cells.
2. For frozen cells: spin in eppendorf with warm medium and wash with PBS.
3. Collect into tubes and spin down at 300 G for 5 min.
4. Resuspend in 200 μL of PBS + 20 μL **proteinase K** + 20 μL **RNAse A**.
5. Vortex and stand for 2 min at RT.
6. Add 200 μL **lysis/binding buffer**.
7. Vortex and incubate at 55 °C for 10 min.
8. Add 200 μL ethanol.
9. Vortex for 5 s. [and optional: freeze at -20 °C or -80 °C]

**Binding**

1. Add each sample (640 μL) to spin collection tubes & centrifuge at 10,000 G for 1 min.
2. Discard flowthrough & place on new tubes.

**Washes**

1. Add 500 μL **wash buffer 1** & centrifuge at 10,000 G for 1 min.
2. Discard flowthrough & place on new tubes.
3. Add 500 μL **wash buffer 2** & centrifuge at 20,000 G (max speed) for 3 min.
4. Discard flowthrough & place on 1.5 collection tubes.

**Elution**

1. Add 50 μL nuclease-free water to center of column and stand for 1 min.
2. Centrifuge at 20,000 G (max speed) for 1 min.
3. Re-add 50 μL flowthrough to center of column and stand for 1 min.
4. Centrifuge at 20,000 G (max speed) for 1.5 min.
5. Keep sample for quantification or freeze.

**Qubit DNA quantification**

1. Set up required tubes: n samples + 2 standards.
2. Prepare working solution: 0.2 \* (n+2 +2 extra) mL of dsDNA HS buffer + 1/200th of dsDNA HS reagent.
3. Add 199 μL of reagent to sample tubes and 190 μL to standard tubes.
4. Add 1 μL per sample and 10 μL of standard 1 & 2.
5. Vortex for 5 s and stand at RT for 2 min.
6. Run samples in Qubit fluorometer: dsDNA High Sensitivity, read standard 1, read standard 2, read samples. Lid must be closed during readings. Note down values one by one.
7. Mouse genotyping

**Ear notching**

1. Notch mouse ears in the order L, R, LR, LL, RR. Alternatively, request procedure on Pyrat (QS cages, Work request, Procedure, Ear punch wo anaesthesia).
2. Store ear notches at -20 °C until ready to process.

**Sample preparation**

1. Prepare lysis buffer using molecular biology grade reagents:

|  |  |  |
| --- | --- | --- |
|  | For 50 mL: | Final [ ] |
| 1 M Tris pH 8.0 | 2.5 mL | 50 mM |
| 5M NaCl | 0.2 mL | 20 mM |
| 10% SDS | 0.5 mL | 0.1% |
| Ultra pure H2O | 44.8 mL |  |

1. Prepare complete lysis buffer: 200 μL per sample + 10% in 1.5 mL tubes, including 4% of 20 mg/mL **proteinase K** (Thermo Scientific EO0492) (e.g. 3.84 mL + 160 μL for 20 samples).
2. Pulse centrifuge the biopsies before opening the tubes.
3. Add 200 μL of complete lysis buffer per tube and incubate shaking (600 rpm) either 2 h at 55 °C (or overnight at 37 °C). Check that tissues are fully lysed by flicking each tube.
4. Incubate for 10 min at 95 °C to inactivate proteinase K.
5. Centrifuge at 10,000 G for 5 min.

**PCR**

1. Dilute 1 μL of the lysate in 15 μL of TE buffer, store the rest at -80 °C.
2. Prepare mastermix for each gene, with 10% excess volume. Per well: 5 µL SYBR green, 0.2 µL primer mix, 2.8 µL RNAse-free water. Vortex SYBR green/mix before pipetting!
3. Add 2 µL of cDNA samples to 384 well plate (controls: 2 µL water).
4. Spin down plate and add 8 µL of mix to plate.
5. Cover plate with optical film.
6. Insert plate in PCR machine, open software and select program: CT, SYBR green, **standard**, low ROX, 10 µL volume, default cycles with **melting curve**; and input samples/targets for each well.
7. Run samples and save eds file when prompted.
8. After running, check amplification curves and melting curves, and adjust Ct thresholds if necessary.
9. Export sample setup, amplification data and results as xls.
10. Run 1% agarose gel to check size if necessary.

MasterMixes:

Perfecta ToughMix (no Sybr, ROX, no loading dye)

BioLabs OneTaq w/standard buffer M0482 (no Sybr, no ROX, no loading dye)

DreamTaq Hot Start Green Master mix K9021 (no Sybr, no ROX, green dye)

RedTaq Ready Mix R2523 (no Sybr, no ROX, red dye)

1. Mycoplasma screening

**Sample preparation**

1. Collect 1 mL of supernatant from >3 day cultures in Eppendorfs
2. Spin supernatant down at max speed (>10,000G) for 5 min
3. Remove most of the supernatant leaving 100 µL in the tube
4. Resuspend the pellet by vortexing the sample

**qPCR**

1. Dilute probe 1 in 10 in appropriate volume of RNAse free water, see table below.
2. Create PCR master mix for 2x(number of samples) + 2x(3 positive standards) + 2 medium + 2 water + 2 extra = 2x(number of samples) + 12

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Stock conc | Working conc | Final conc | μL/sample | μL/(2x (20 samples) + 12) |
| PerfeCTa Toughmix Low ROX | 2x | | 1x | 5 μL | 260 μL |
| Forward primer (GPO3) 5'-GGGAGCAAACAGGATTAGATACCCT-3' | 10 μM | | 1 μM | 0.2 μL | 4.4 μL |
| Reverse primer (MGSO) 5'-TGCACCATCTGTCACTCTGTTAACCTC-3' |
| MGB Probe 5'-FAM-TAGTCCACRCYGTAAACGAT-MGB-3' | 100 μM | 10 μM | 0.25 μM | 0.25 μL | 5.5 μL |
| Nuclease-free water | - | - | - | 7.8 μL | 51.7 μL |

Primer ref: <https://doi.org/10.1128/aem.59.2.655-.1993>; probe is degenerate and a new design.

7. Pipette 8 μL master mix per well, spin down plate at 550 G for 1 min.

8. Pipette 2 μL sample per well, spin down plate at 550 G for 1 min.

9. Cover plate and run in machine (standard mode): 2 min at 50°C, 10 min at 95°C and 40 cycles of 30s at 95°C, 30s at 55°C and 1 min at 72°C.

**OLD METHOD**

**PCR**

5. Create PCR master mix for number of samples + 2 (pos and neg) + 10% excess.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Stock conc | μL/sample | μL/(20+2) samples |
| BioLabs OneTaq w/standard buffer M0482 | 2x | 10 μL | 220 μL |
| Forward primer (GPO3) 5'-GGGAGCAAACAGGATTAGATACCCT-3' | 100 μM | 0.1 μL | 2.2 μL |
| Reverse primer (MGSO) 5'-TGCACCATCTGTCACTCTGTTAACCTC-3' | 100 μM | 0.1 μL | 2.2 μL |
| Nuclease-free water | - | 7.8 μL | 171.6 μL |

Primer ref: <https://doi.org/10.1128/aem.59.2.655-.1993> (see also probe sequence 5'-CTTAAAGGAATTGACGGGAACCCG-3')

6. Pipette 2 μL of each sample, including negative control (water) and positive control (myco+ DNA extracted samples) into PCR tube strip.

7. Add 18 μL of master mix per tube.

8. Run PCR on MMJ Thermal cycler (Program Main\MC) for 2h:

- 35 cycles of (30s at 95°C, 30 s at 55°C, 60 s at 72°C) then 4°C hold forever

**Gel electrophoresis**

9. Make a 1% agarose gel, for a large cassette: 2.5 g agarose in 250 mL 1x TBE.

10. Heat solution in microwave to dissolve agarose.

11. Leave agarose to cool down, then add 3 μL Gel Red per 100 mL (i.e. 7.5 μL).

12. Add stained agarose to cassette and leave to set.

13. When it is ready, remove plastic comb and fill tank with 1x TBE.

14. Prepare samples by making 1.5 μL drops of loading dye on parafilm.

15. Pipette 4 μL of HyperLadder 50bp into the first well of each row.

16. Mix 9 µL of PCR product each drop of loading dye and pipette 9 μL of the mix into gel.

17. Run gels at 120V (small) or 200V (large) for at least 20 min.

Store samples and PCR products for 6 months.

1. Reporter plasmid transfection

**Cationic lipid transfection**

1. Pretreat cells if necessary.

2. Trypsinise cells and replate in **white clear bottom 96 well plate** with 15,000 cells/well in 100 μL.

3. Incubate for 18 hours at 37°C in 5% CO2.

4. Prepare **transfection mix** (8 μL/well for n + n/10 wells). E.g. for 96 wells:

Total volume: 8\*110 = 880 μL

* Ham-12 no serum medium: 880 – 12 – 36 = 832 μL
* Firefly plasmid: 0.1 μg \* 1 μL/μg stock plasmid \* 110 wells = 11 μL
* Renilla plasmid (pGL4.75[hRluc/CMV]): 0.01 μg \* 1 μL/μg stock plasmid \* 110 wells = 1.1 μL
* FuGene HD: 12 \*34 = 36 μL

5. Add 8 μL transfection mix per well and incubate for 18 hours at 37 °C in 5% CO2.

**Electroporation**

1. Start 4D-Nucleofector System and select respective vessel type.

2. Create or upload experimental parameter file and select the appropriate Nucleofector Solution (P3) and Program (EH 100).

3. Prepare cell culture vessels with desired volume of recommended culture media and pre-incubate vessels in a cell culture incubator.

4. Prepare **transfection mix** and leave at room temperature. For 100 μL cuvette:

* Firefly plasmid (1 μL/μg stock): 2 – 9 μL
* Renilla plasmid (pGL4.75[hRluc/CMV]) (1 μL/μg stock): 0.2 – 1 μL
* Ingenio electroporation solution: 90 μL

5. Harvest the cells by trypsinization and count.

6. Centrifuge the required number of cells at 200 G for 5 minutes (or 100 G for 10 min), at RT.

7. Resuspend the cell pellet carefully in room temp. transfection mix (0.5 – 1 million per 100 μL) and pipette into nucleocuvette – tap to get rid of bubbles and make sure the sample covers the bottom.

8. Place nucleocuvette with closed lid in the retainer of the 4D-Nucleofector X Unit, facing the front.

9. Start nucleofection process by pressing the “Start” on the display of the unit.

10. After run completion (1 s), take nucleocuvette to TC and leave for 3 min (RPMI) or 10 min (DMEM).

11. Resuspend cells with pre-warmed medium, mix and plate 10 – 100k cells/well in white 96 well μclear plate.

12. Incubate for 24 hours at 37 °C in 5% CO2.

**Treatment**

6. Prepare treatment in medium for all conditions, including control (35 μL/well):

Total volume: 15 wells \* 35 μL/well = 525 μL

FMED = 500 μL

5% CSE = 26.2 μL

7. Change medium in plate and incubate for 18 hours.

**Luminescence readings**

8. Take plates out of incubator and leave at RT for 15 min.

9. Prepare firefly luciferase substrate solution (1 bottle buffer + 1 bottle substrate power) – aliquot and store at -80C.

10. Add same volume (35 μL/well) of firefly luciferase substrate solution.

11. Incubate at RT for 10-60 minutes, checking that cells are lysed.

12. Read luminescence at **560 nm**.

13. Prepare stop & glo renilla solution (1:100 substrate in buffer).

14. Add same volume (35 μL/well) of renilla luciferase substrate solution.

15. Incubate at RT for 10-60 minutes.

16. Read luminescence at **500 nm**.

1. Stable transduction

**Lentivirus generation**

1. Vectors
2. HEK cells
3. Spin down supernatant to isolate virus

**Transduction**

Day 1.

1. Prepare feeders in T25s, minimum 3 flasks: no virus, control virus (shRNA pLKO.1), virus (shRNA p53).

Day 2

1. Collect conditioned medium from basal cells & filter.
2. Seed 150,000 basal cells/T25 in 50% conditioned/50% normal medium.

Day 3

1. Add transduction medium for 7 h: 2 mL FMED + 4 μg/mL polybrene per flask + 30 μL virus per flask
2. Remove medium and add 4mL FMED per flask. Waste in trizol empty bottle disposal.

Day 4

1. Change medium again.

Day 8

1. Split cells and reseed with antibiotic, or keep in flask with antibiotic if not confluent.
   1. blasticidine (4 μg/mL)
   2. puromycin (0.7 μg/mL)
2. Keep with antibiotic until no-virus-control cells are all dead (puromycin: add for 3 days, change medium & readd for 2 days).

**Antibiotic** **selection**

Do antibiotic selection after splitting (freeze the rest), 72 hours later?  
Aliquot blasticidine and freeze, keep the one to use always on ice in fridge  
- add blasticidine 4ug/ml  
- control no virus flask: once dead selection is finished (5 days)  
  
Harsher selection maybe better  
Western, if, qpcr for tp53

1. Comet assay

**Slide preparation**

1. Add circular spacers to slide.
2. Prepare 1% agarose in distilled water.
3. Pipette agarose onto the circles to form a base layer, dry at 37 °C in oven overnight.

**Cell treatment**

1. Prepare 0.8% **LMP agarose** in PBS or distilled water, keep at 50-60 °C.
2. Treat cells with 0.5 mM H2O2 for 1 h.
3. Prepare 1 L of **alkaline solution** in glass bottle and store in cold room

* 12 g NaOH (0.03 M final conc.)
* top up to 1 L with distilled H2O
* 2 mL EDTA solution (500 mM stock; 1 mM final conc.)

1. Prepare 50 mL of **lysis buffer** in falcon and store in fridge

* 7.3 g NaCl (2.5 M final conc.)
* 10 mL EDTA solution (500 mM stock; 100 mM final conc.)
* 5 mL lysis solution (10X stock, ab238544) [alternative: 1 mL of 100% triton X100]
* 5 mL of DMSO
* top up to 45 mL with distilled H2O
* add 5 M NaOH dropwise to pH 10
* top up to 50 mL with distilled H2O

1. Trypsinize cells and resuspend in 300 μL LMP agarose, then pipette 100 μL onto the circle (fast or else it will set). Flatten with a coverslip (messy).
2. Put slides in plastic box at 4°C for 15 minutes.
3. Add 5 mL lysis buffer per box and leave at 4°C for 15 min.
4. Wash slides with alkaline solution and leave at 4°C for 30 min.

**Electrophoresis**

1. Prepare electrophoresis chamber in large plastic **ice box**.
2. Put slides in electrophoresis chamber and fill with cold alkaline solution so that it just covers them.
3. Run at 300 mA constant current for 22 minutes.
4. Wash with cold distilled water three times for 2 min.
5. Wash with cold 70% ethanol for 5 min.
6. Air dry overnight with a dessicant sachet in the box.

**Staining**

1. Add 100 μL of 1X GelRed in PBS for 15 min.
2. Wash with PBS
3. Add 20 μL of PBS or antifade mountant per circle and coverslip with nail polish.
4. Image at 10X on the 525 channel on an epifluorescence microscope.
5. Analyse images on ImageJ with OpenComet plugin (<https://cometbio.org/download_links.html>)

Proteins

1. Western blot

**Protein extraction**

1. Spin down cells in 15 mL Falcon tubes, discard supernatant and add **RIPA buffer**.
2. Vortex and leave on ice for 10 min three times. Last vortex should be longer (30 sec).
3. Spin at max speed for 20 min at 4C and keep supernatant.
4. Aliquot samples for quantification (e.g. 3 µL protein + 12 µL water, 1 in 5).

**Protein quantification**

1. Prepare BSA standard stock (15 µL) from BCA stocks (glass mini containers).

|  |  |  |
| --- | --- | --- |
| Concentration (µg/µL) | BSA volume (2 µg/µL) | Water volume |
| 0 | 0 | 16 |
| 0.125 | 1 | 15 |
| 0.25 | 2 | 14 |
| 0.5 | 4 | 12 |
| 0.75 | 6 | 10 |
| 1 | 8 | 8 |
| 2 | 16 | 0 |

1. Mix **working reagent** (**A** (white):**B** (blue)= 50:1, i.e. 1 mL:20 µL).
2. Add 5 µL of each standard and sample to Greiner plate (flat bottom, black) in triplicate.
3. Add 200 µL of working reagent to each well, pipette up and down, and shake plate.
4. Incubate for 30 min at 37 °C.
5. Put plate into Fluostar Optima microplate reader. Open Optima program, select test protocol, absorbance mode, BCA, endpoint, Greiner FB, select standards and samples, input standard concentrations, ok, measure (1 min).
6. Export results to xlsx and save file using Optima Data Analysis.

**Gel electrophoresis** *(for Bio-Rad Mini-PROTEAN, also applicable to Invitrogen XCell SureLock)*

1. Calculate protein, buffer and water volumes needed. Wait for protein to thaw.
2. Prepare **loading buffer** (stock kept in box): 50 µL mercaptoethanol (hood) + 1 mL NuPage (polyacrylamide).

Alternative: Laemmli buffer (incl. SDS).

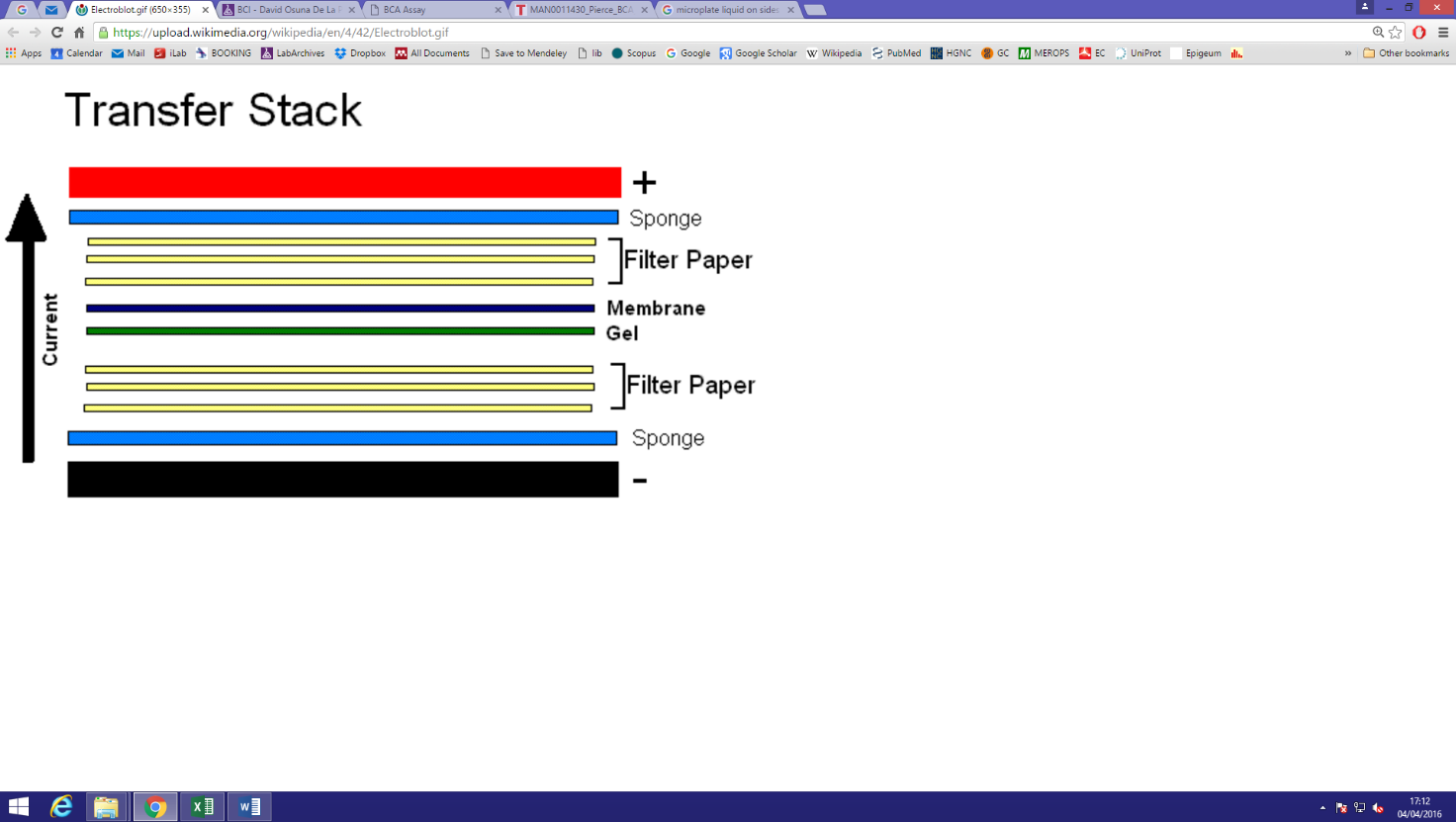
1. Pipette protein, buffer and water into eppendorfs and leave on Eppendorf block for 10 min at 95°C.
2. Prepare **MOPS buffer**: 950 mL water + 50 mL 20x MOPS. Alternative: MES.
3. Select gel (main fridge 1), remove bottom strip, slide off top, check well integrity (correct if necessary with pipette tip).
4. Set up tank and fill electrode chamber with MOPS buffer to check leaks. Make sure electrode colours match!
5. Pipette ladder (1st bench fridge) and protein solution into wells using ultra-thin pipette tips, fill empty lanes with buffer!
6. Connect cable to BioRad apparatus, select manual, 100 V, run for 1 h until 40 kDa band reaches the bottom of the gel. When finished turn off, disconnect, and take out gel.

**Membrane blotting**

1. While it is running, cut 6-10 pieces of filter paper a little larger than the gel, and 1 piece of Hybond C extra (nitrocellulose) membrane the same size as the gel.
2. Prepare **TBST**: 900 mL water + 100 mL 10x TBS + 1 mL tween. Cap tip to pipette tween, leave tip in cylinder, add stirrer and stir for 30 min.
3. Prepare **transfer buffer**: 700 mL water + 200 mL methanol (PVDF) or ethanol (nitrocellulose membrane) + 100 mL 10x tris-glycine.
4. Soak the filter papers, the membrane and the cassette sponges in transfer buffer in a small container for at least 5 minutes while setting up the transfer apparatus.

*Wet transfer*

1. Place in the transfer cassette: sponge - 3 layers of filter paper - the gel - the membrane on top of the gel - 3 layers of filter paper - sponge. Roll over layers at the end to remove air bubbles in only one direction. Seal the transfer cassette and fill with transfer buffer.



1. Immerse the transfer apparatus on ice, run at 20 V for 1 h (or 2 h if necessary).

*Semi-dry transfer*

1. Place in the BioRad trasblot turbo compartment with just some transfer buffer: 5 layers of filter paper - the membrane - the gel on top of the membrane - 5 layers of filter paper. Roll over layers at the end to remove air bubbles in only one direction. Seal and close the transfer compartment.
2. Set mode StandardSD 25V 1A for 30 min.
3. Prepare **5% BSA**: 2.5 g BSA (measure on falcon) (main fridges) + 50 mL TBST. Leave on rocker at 4°C.
4. Remove the membrane, mark an edge for orientation, wash once with TBST [and: optional add Ponceau’s to check protein bands, wash three times with TBST].

**Antibody staining**

1. Add **5% BSA** and shake for 1 hour at RT on rocker.
2. Wash with TBST, and add primary antibody (1st bench fridge) (diluted 1:1000 in 2.5% BSA**:** 1 in 2 solution of 5% BSA in TBST), i.e. 7 µL in 7 mL BSA per gel. Leave on rocker at 4°C (fridge) overnight.
3. Wash with TBST for 5 min at RT three times.
4. Add secondary antibody (diluted 1:1000 in 2.5% BSA), shake 1 hour at RT.
5. Wash with TBST for 5 min at RT three times.
6. Take out **ECL substrate** solutions (main fridge 1) and leave for 10 min at RT. Then mix solution 1 and solution 2 1 to 1 (e.g. 0.7+0.7 mL) so that the final volume is 0.1 mL/cm2 of membrane. Leave on the membrane for 5 min.
7. Drain ECL solution (into sink and onto paper), and place membrane on black tray (chemiluminescence) into Amersham Imager (“ChemiDoc”). Expose for 1 minute, every 15 seconds. Select image(s) and save to Cross Dept Share. Repeat if necessary with increased or decreased times.

**Antibody re-staining**

1. To re-develop a blot, wash in TBST and re-add ECL solution. If signal is too faint (e.g. membrane older than 1 week), re-add secondary antibody.
2. To check for a different protein strip antibodies by adding 0.2-0.5 M **NaOH** for 5-10 min (no longer!). Wash three times with TBST and add new primary (different species!) [if membrane is clean and proteins are of very different sizes stripping is not necessary].

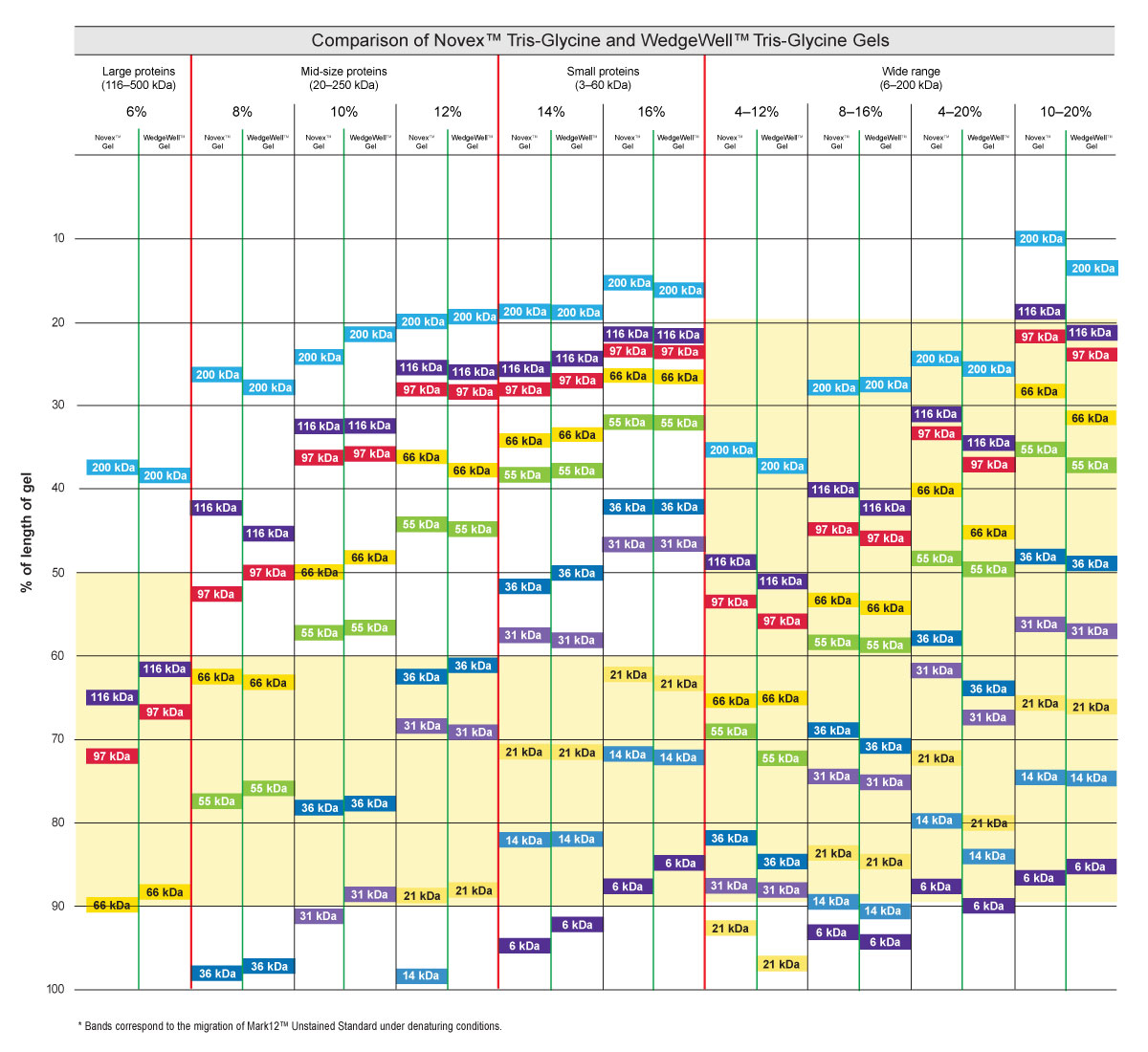
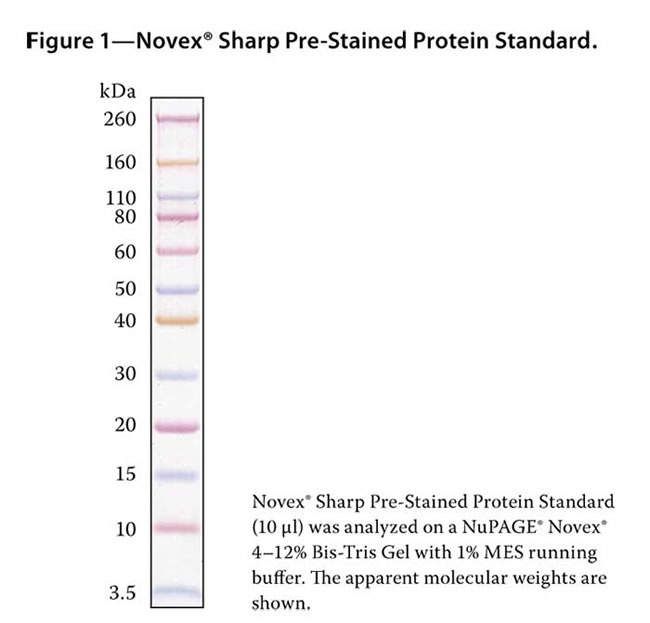
**Solutions**

10X TBS: 24.23 g tris (Trizma base) + 80.06 g NaCl into 800 mL dH2O. Bring to pH 7.6 with high conc. HCl. Top up to 1 L.

10X tris-glycine: 30 g tris (Trizma base) + 144 g glycine + make up to 1 L dH2O.

0.5 M NaOH: 2 g NaOH + 100 mL dH2O.

Novex gels Novex ladder on 4-12 bis-tris gel

1. Organelle isolation

ER & mitochondria: https://pmc.ncbi.nlm.nih.gov/articles/PMC4607254/

1. Label-free proteomics
2. Histology

**Tissue processing**

1. Place tissue into a cassette of the appropriate size with one sponge on either side, label with pencil sample ID and name.
2. Place cassettes into metal rack in tissue processor and close lid.
3. Select programme (short 8 h or long 16 h) and run.

**Tissue embedding**

1. Turn on embedding station and wait for wax to melt.
2. Take cassettes from tissue processor and place in wax bath.
3. Run cleaning programme in tissue processor (1 h).
4. Place tissue in metal tray of the appropriate size, with cassette lid on top.

**Tissue cutting**

1. Pre-chill blocks at -20C overnight.
2. Place blocks on chilled surface at -7°C, take blades and superfrost plus slides, fill water bath with tap water.
3. Place block in microtome holder, adjust angles and distance.
4. Place blade in microtome and start moving block forward at 20 μm steps.
5. Once being cut, go as deep as necessary and switch to 5 μm.
6. Cut ribbons and pinch with tweezers.
7. Place ribbons in water batch and pick with slide, ensuring it attaches on the upper side.
8. Label slide and leave out to dry overnight.

**H&E staining**

1. Open autostainer, remove all lids, open water tap.
2. Select program.
3. Put slides in two trays with holders, ensuring the upper side if facing the right way, and place in correct site (S1 or S2).
4. Run program for 1 h.
5. Take trays and place in coverslipper, and place empty tray in outlet.
6. Run xylene test and run a bit of tape.
7. Run all slides.
8. Check coverage and cut extra tape if necessary, adding a drop of xylene for binding.
9. Sudan black staining

<https://link.springer.com/protocol/10.1007/978-1-4939-6670-7_10>

<https://www.protocols.io/view/an-optimised-protocol-for-the-detection-of-lipofus-b94sr8we.html>

combine protocols into one

1. Immunofluorescence staining

*All values for 96 well plate.*

*All washes: place pipette against the side of wells for each, be careful of lifting up the matrix with pipette*

1. **Fixation:** Aspirate media and wash in PBS x1. Immediately fix structures in 200 µL of 4% paraformaldehyde for 10-20mins at RT.

**Senescence staining**

1.1 Wash with 1% BSA.

1.2 Stain for senescence (1:1000) for 2 h at 37 ºC without CO2.

1.3 Wash 3x with PBS.

**8-oxo-G staining** ([1](https://pmc.ncbi.nlm.nih.gov/articles/PMC9943661/#SEC2), [2](https://pmc.ncbi.nlm.nih.gov/articles/PMC2875005/#sec2))

1.4 Denature DNA with 2N HCl for 30 min at 37 ºC.

1.5 Neutralize with 0.1 M Tris HCl pH 8 for 20 min.

1.6 Wash 3x with PBS.

1. **Permeabilisation**: For 10 minutes at RT 0.25% Triton X-100 in PBS.
2. **Wash:** 1x for 10 minutes with IF wash buffer at RT.
3. **Blocking:** For 1 hour incubate with 200ul of blocking solution (). *Can be blocked overnight / weekend at 4 degrees.*
4. **Primary antibody:** Remove the blocking solution and incubate with primary antibody diluted in 200 µL of blocking solution overnight at 4 degrees. Cover with parafilm and foil.
5. **Wash:** 3x for 10mins with IF wash buffer at RT.
6. **Secondary antibody:** incubate with secondary antibody diluted in blocking solution for 1 hour at RT (1:1000-1:500 dilution for most). Cover with parafilm and foil.
7. **Wash:** 3x for 20 mins with IF wash buffer at RT.
8. **Nuclear stain:** Incubate with 1x DAPI in PBS for 5-10mins at RT.
9. **Final wash:** Wash and leave in PBS or, if mounting, wash 1x for 10 mins with PBS.
10. **Mount slide:** using ProLong Antifade and allow to dry overnight at RT. Can be stored at 4 degrees for a few months, or long-term at -20

**Blocking solution (easy) =** TBST, 1% BSA

**IF wash buffer (easy)** = TBST, 0.1% BSA

**Blocking solution =** IF Wash Buffer + 10% Goat serum

**IF wash buffer** = PBS, 0.1% BSA, 0.1% Triton X-100, 0.05% Tween-20, adjusted to pH 7.4.

Senescence exp 9

A SD-488 - block - rb p21 1:500 (my box) - wash - antirb 555 (Kate yellow box) + phal 647 (Kyren Box 1) - wash - DAPI - PBS

B SD-488 - block - rb p16 1:250 (my box) - wash - antirb 555 (Kate yellow box) + phal 647 (Kyren Box 1) - wash - DAPI - PBS

C block - rb Ki67 ab16667 1:250 (Kyren Box 1) + ms Krt5 ab17130 1:500 (Kate blue box) - wash - antirb 488 (Kate yellow box) + antims 555 ab150106 (Kate blue box) + phal 647 - wash - DAPI - PBS

Senescence exp 15

A SD-488 - block - rb p21 1:500 (my box) - wash - antirb 555 (Kate yellow box) + phal 647 (Kyren Box 1) - wash - DAPI - PBS

B SD-488 - block - rb p16 1:250 (my box) - wash - antirb 555 (Kate yellow box) + phal 647 (Kyren Box 1) - wash - DAPI - PBS

C SD-488 - block - rb Ki67 ab16667 1:250 (Kyren Box 1) + ms Krt5 ab17130 1:500 (Kate blue box) - wash - antirb 555 (Kate yellow box) + antims 647 ab150106 (Kate yellow box) - wash - DAPI - PBS

all secondaries 1:500

Epi exp 4

A block - rb p21 1:500 (my box) - wash – WGA 488 (1:200) + antirb 555 (Kate yellow box) + phal 647 - wash - DAPI - PBS

B block – ms IgG3 KRT14 1:500 (my box) + rb Ki67 1:250 (my box) + ms IgG1 KRT5 (Kate’s blue box) - wash – antims IgG3 488 + antirb 555 (Kate yellow box) + antims IgG1 647 - wash - DAPI – PBS

Sen exp 44 (density assay)

A block - ms IgG3 KRT14 1:500 (my box) + rb p21 1:500 (my box) – antims H+L 488 + antirb 555 (Kate yellow box) + phal 647 - wash - DAPI - PBS

Sen exp 45 epi

P681 block - rb Ki67 1:250 (my box) - wash – WGA 488 (1:200) + antirb 555 (Kate yellow box) + phal 647 - wash - DAPI – PBS

P682 block - rb p21 1:500 (my box) - wash – WGA 488 (1:200) + antirb 555 (Kate yellow box) + phal 647 - wash - DAPI - PBS

P683 block – ms IgG3 KRT14 1:500 (my box) + rb activated Notch1 1:500 (my box) + ms IgG1 KRT5 (Kate’s blue box) - wash – antims IgG3 488 + antirb 555 (Kate yellow box) + antims IgG1 647 - wash - DAPI - PBS

Epi P695

P695 block - ms IgG1 MUC5AC 1:500 + ck Krt5 ab17130 1:500 (Kate blue box) - wash – antims IgG1 488 (Kate blue box) + antick 555 ab150106 (Kate blue box) + phal 647 - wash - DAPI – PBS

Epi exp 7

P702 block – ms IgG2b AcTub 1:500 + ms IgG1 MUC5AC 1:500 + ck Krt5 905901 1:500 (Kate blue box) - wash - antims IgG2B 647 + antims IgG1 488 + antick 555 ab150106 (Kate blue box) - wash - DAPI – mount

Epi exp 8

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Channel | Sen exp 9 A | Sen exp 9 B | Sen exp 9 C | Sen exp 9 A |  |  |  |  |  |
| 355/450 | DAPI | DAPI | DAPI | DAPI | DAPI | DAPI | DAPI | DAPI | DAPI |
| 488/530 |  |  |  |  |  |  |  |  |  |
| 555/565-594/614 |  |  |  |  |  |  |  |  |  |
| 647/671 |  |  |  |  |  |  |  |  |  |
| Channel | Epi 8 ASC314 6wp A | B | C | D | Epi 9 P709 24wp | new |  |  |  |
| 355/450 | DAPI | DAPI | DAPI | DAPI | DAPI | DAPI | DAPI | DAPI | DAPI |
| 488/530 | MUC5AC ms-IgG1 | p21 ms-IgG1 | SCGB1A1 rb | Krt5 rb | Krt5 ms-IgG1 DNW | Krt5 rb |  |  |  |
| 555/565-594/614 | acTub ms-IgG2b | WGA-594 | TFCP2L1 goat | P53 ms-IgG2b | Ki67 rb | Krt13 ms-IgG |  |  |  |
| 647/671 | Krt5 rb | p63 rb | Krt5 ms-IgG1 | Phal-647 | p21 ms-IgG2a | Phal-647 |  |  |  |
| Channel | Epi exp 70 A | B | C | D | Epi exp 71 |  |  |  |  |
| 355/450 | DAPI | DAPI | DAPI | DAPI | DAPI |  |  |  |  |
| 488/530 | TRF1/2 ms | pS15p53 ms | SD | SD | TRF1/2 ms |  |  |  |  |
| 555/565-594/614 | gammaH2AX rb | TP53BP1 rb | p21 rb | DCR2 rb | TP53BP1 rb |  |  |  |  |
| 647/671 | Phal-647 | Phal-647 | Krt5 ms | Krt5 ms | Phal-647 |  |  |  |  |

Probes

Iron 646 1:1000 1 mM (50 nmol recon in 50 uL dmso) to 1 uM  
Mitotracker green fm 488 50 ug 1 mM stock 1:10000 - 100 nM  
Hoechst 10 mg/mL 1:10000 - 1 ug/mL

1. Confocal imaging

Z stacks

In thick tissue: Auto Z brightness correction: extrapolate and interpolate

Highest point with tdTom

Center point

Lowest point with tdTom

Tilescan

1. Immunohistochemistry staining

**Day 1**

1. Dewax slides (in plastic holder) using the autostainer (instructions on instrument). Plastic tray, 20 min.
2. *While dewaxing*: Prepare 10 mM citrate buffer: 2.94 g of trisodium citrate, 0.5 mL tween-20 in 900 mL dH2O, top up to 1000 mL and add 200-400 μL 0.5 M HCl to pH 6 (check with strips or pHmeter).
3. Wash 2x with PBS (communal histology bottle). Plastic box (in histology room cabinet), 10 min.
4. Immerse slides in citrate buffer. Plastic box, 1 min.
5. Microwave at 100% power. Plastic box, 10 min.
6. Top up citrate buffer, microwave at 40% power. Plastic box, 5 min.
7. Cool down slides on bench. Plastic box, 20 min.
8. *While cooling*: Prepare a humidity chamber by placing wet absorbent paper towels inside a metal box (in histology room cabinet).
9. *While cooling*: Prepare blocking buffer: 5% serum (from secondary ab species), 1% BSA, 0.1% triton X100 in PBS.
10. Wash 2x with PBS. Plastic box, 10 min.
11. Flick off PBS and draw around tissues with hydrophobic pen (don’t let them dry out). Humidity chamber, 5 min.
12. *If using primary antibodies made in mouse on mouse tissue*, incubate tissues in MOM block (Vector; dilute 1 drop of stock solution in 1mL of PBS) at 4°C overnight. Wash 3x times with PBS. 10 min
13. Incubate each slide with 100 μL of blocking buffer at RT. Humidity chamber, 1 h.
14. *While blocking*: Prepare primary ab solution in blocking buffer: usually 1:50 - 1:500.
15. Incubate each slide with 100 μL of primary ab solution at 4°C. Humidity chamber, overnight.

**Day 2**

1. Prepare PBST: 0.1% triton in PBS. Humidity chamber, 5 min.
2. Wash 2x with PBST. Humidity chamber, 20 min.
3. *While washing*: Prepare secondary ab solution in blocking buffer: usually 1:1000.
4. Incubate each slide with 100 μL of secondary ab solution at RT. Humidity chamber, 2 h.
5. Wash 2x with PBST. Humidity chamber, 20 min.
6. Stain with DAPI (1:10,000 already dilute solution) and phalloidin (1:1000 in DAPI solution). Humidity chamber, 30 min.
7. Wash 2x with PBST. Humidity chamber, 20 min.
8. Wash slide in PBS (in Falcon) and then dH2O (in Falcon) to remove salts, then remove dH2O. 5 min.
9. Add 1 drop (40 μL) Fluoromount G anti-fading mounting medium on each tissue, put coverslip on top and seal the edges of with nail polish. 5 min.
10. Dry in the dark at RT; then store at 4C. Dark box, 45 min.
11. Magnetic separation

**Cell dissociation**

1. Cut tissue and incubate at 37 °C for 1-2h in a solution with 5 mL medium, 100 μL collagenase XI and 100 μL dispase.
2. Prepare running buffer:

Add 372 mg EDTA to 500 mL PBS bottle to make 2 mM EDTA.

Prepare two 50 mL falcons, each with 50 mL buffer + 250 mg BSA or 250 μL FBS. Keep on ice. Ideally degas using bottle with vacuum connector.

1. Filter digestion through 100 μm filter, spin down.

**Staining**

1. Prepare staining solution: 300 μL buffer, 50-100 μL Fc Block and 100 μL Ab-Microbeads.
2. Stain pellet for 1 h on ice.

**Magnetic separation**

1. Spin down and 6 mL (or 1mL) of buffer.
2. Run 3 mL (or 500 μL) through LS columns on MultiMACs (or MS column on MiniMACS) magnet with stand , with two 15 mL falcons underneath, to condition column. Discard eluent.
3. Run 3mL (or 500 μL) of sample in each column into falcons.
4. Add 4 times 3mL (or 500 μL) of sample in each column until falcons are filled. This is the negative population.
5. Remove column from stand, place on falcon and add 5 mL (or 1 mL) of buffer, press plunger to release all positive cells. Repeat steps 7-10 for a more pure population.
6. Spin down cells, resuspend in media and plate.
7. Flow cytometry

**EdU**

1. Add 10 μM EdU for 24 h (3-36 h) (stock 10 mM: add 4 mL PBS; dilute 1:1000).

**CellROX**

2. Add NAC for 1 h to negative ctrls (250 mM stock: 10 mg in 245 μL; dilute 1:1000 for 25 μM final)

3. Add TBHP for 1 h to negative and positive ctrls (50 mM stock: 3.22 μL in 496.8 μL PBS; dilute 1:250 for 200 μM final)

4. Add CellROX reagent for 1 h (250 μM stock: 7 μL reagent + 63 μM DMSO; dilute 1:500 for 500 nM final)

**Cell dissociation**

5. Remove medium from plate and dissociate cells with TrypLE (or trypsin/EDTA) for 10-20 minutes. For macrophages, use Accutase.

6. Transfer to Eppendorf or Falcon and spin down, add 200 μL PBS and transfer to V-bottom plate (unstained, single stain controls, FMOs, etc.).

(7. Prepare viability control by heating unstained cells in heating block >65°C and putting at 4°C on ice.)

8a. Wash with PBS once. Keep on ice.

8b. For macrophages, dilute TruStain FcX in PBS 1:20 and add 50 µL per well for 10 min.

**Membrane stain**

9a. Prepare staining solution:

- viability dye solution by diluting 1:1000 in PBS **or** DAPI by diluting 5 mg/mL stock 1:5000 in PBS

- membrane antibodies in same solution (1:20 – 1:100).

9b. Stain cells in tubes/plates with 100 µL viability dye solution for **30 min** [**1 h with abs**] on ice, dark.

9c. Stain one drop of UltraComp beads with 5 μL of antibody and one drop of ArC positive beads with 3 μL viability dye for 30 min, then add 2 mL medium, spin down and resuspend in 150 μL PBS (and add one drop ArC negative beads).

10a. Prepare fixing solution (4% PFA in PBS) and washing solution (1% FBS in PBS).

10b. Wash cells with 1% FBS.

10c. Fix cells with 4% PFA for **10 min** (for senescence, CellROX, EdU, intracellular and next day).

10d. Wash cells with 1% FBS.

**Senescence stain**

11a. Prepare senescence dye solution by diluting probe 1:1000 in senescence buffer.

11b. Incubate cells with senescence dye solution for **1.5 h** at 37 °C without CO2! (in oven) in the dark.

11c. Wash cells with 1% FBS.

**Intracellular stains**

12a. Permeabilise cells with 0.25% triton X-100 for **5 min** or 10% saponin in 1% FBS in PBS for **10 min**.

12b. Wash cells with PBS.

13a. Add nuclear/cytoplasmic antibodies for **1 h**.

14a. For cell cycle analysis, add 100 µL DAPI for **20 min**.

14b. For EdU staining, make 1X additive by diluting 1:10 in ddH2O, then add 100 μL/well of the following reaction cocktail for 30 min (prepare within 15 min of use):

For 20 samples: 2.2 mL PBS + 50 μL Cu4SO2 + 12.5 azide dye (or 20 μL if too low) + 250 μL additive

15. Resuspend in 150 μL PBS (except EdU) and bring plate, inserts and FACS tubes to FACS room. Ideally run many cells in little volume so that flow can be kept as low as possible.

**Analysis on Sony ID7000**

16. Turn on machine (2 switches on back and power button on front), wake up computer and login into ID7000 software.

17. Start priming, start optics alignment, start daily QC (use Sony aligncheck beads in fridge, shake well before use, add two drops into FACS tube + 450 μL water, place in position 1 (towards door).

18. Create experiment via experiment designer

Notes:

Instrument settings:

Set voltages:

For HBECs, FSC gain: 3, SSC: 4.19

PMT voltages in standard mode, set to max with 0% saturation (**3.58** for **dead cell DAPI and DiI/DiD**).

Enable low dead volume mode (maximizes cells recovered)

Set flow rate (slow 1, fast 8)

Stopping condition: time according to flow rate, then sync condition to all samples.

Change default export folder on preferences

Select dyes (Delete old single stain controls when running new ones, don't combine tandem dyes for dump channel)

TICK ON ALL LASERS AT THE BOTTOM

96 well standard (either bottom type)

1st MUST be UNSTAINED beads:

Unstained ultracomp

Unstained positive arc

Unstained cells\* (to be run again for experiment with higher voltage)

(All fixed as the cells)

Right click move to sample group

Circles are shared worksheets (same gates)

Full color samples run together on autorecord (do it for each group)

Rack/plate in, A1 front left

Saturation stop button select Cells gate (first gate) on samples (not beads) to exclude dead cells

Set current position before preview sample to correct sample

Saturation must be <3%

Sync stopping conditions to apply to all samples

Run samples

Set gates

Unmixing - Unmixing settings

Move unstained cells to single stained controls group and do auto fluorescence analysis

Do not include in unmixing if saturated

Move samples into single stains and back into sample group so UNMIXING is applied

Gate live cells for Unmixing viewer

Decontamination Bleach cleaning and rinse: Facs clean and Water (not rinse). Fill to above rack level.

Export layout, change sample names and import layout

---

Rerun with higher voltage below 3% saturation

---

Turn off unmixing, press auto fluorescence f8nder, select unstained control

Select populations for aurofluorescence adjustment - unmixing settings - aurofluorescence - apply gates

Data export - experiment export

Save FCS file: D / user data / my name

Sony exdat file: Close experiment File / datase / export

---

Open in acquisition to run new samples to saved exp

Click unload plate before opening door

Flowcleaner on flowjo

Panel design

For spectral, similar fluorophores can be used as long as not coexpressed, The rarer the brighter.

For BV dyes, use brilliant stain buffer (blocks ab ab binding) with ab cocktails but not single stain controls or beads. For novafluor dyes use cell block buffer always.

Custom keywords per sample for fcs file R export/analysis.

Control sr file format

Unmixing off for autofluorescence

Copy paste worksheet on sample

Unmixing viewer: click and drag populations left or right to fix mixing errors (but don't do on autofluorescence channels)

Unmixing viewer fluorochrome settings add to library.

Fitc & af488 compatible but in different cell types

Brilliant buffer for bv dyes, but not for beads

Dyes that change fluorescence don't work well in spectral

**Analysis on BD LSRFortessa**

16. Follow instructions on Fortessa, log in into computer and start DIVA software.

17. Create new experiment, cell types and samples, and start gating.

VD: 640 laser 680/60 filter; SD: 488 laser 530/30 filter; DAPI 405 laser 450/50 filter.

18. Run first sample (low speed, run, acquire), and adjust voltages for SSC-A and FSC-A central population.

19. Stop acquiring, standby, do the same to check beads, isotypes, etc.

20. Measure all samples.

21. When finished, right click experiment, export to FCS files. Save in default folder (FSC/Name) folder and copy to OneDrive via browser.

22. Follow cleaning procedure.

23. Log out and turn off computer, machine and power source.

FACS Diva workflow (y / x):

SSC-A / FSC-A – intact cells

FSC-H / FSC-A – single cells

SSC-H / SSC-A – single cells

450-W / 450-A or 640-W / 640-A – single live cells

SSC-A / 582-A (e.g. EPCAM-PE) – cell population of interest

**Cell sorting**

1. Prepare FACS buffer: PBS + 2 mM EDTA (pre-made) + 1% FBS + 10 μg/mL DNAse I

2a. Plan staining and controls (unstained, etc.).

2b. Trypsinise cells and spin down. Stain as above. Resuspend in 500 μL FACS buffer.

3. Pipette into FACS tubes via 40 μm strainer caps.

4a. Prepare 1.5 mL eppendorfs with 1 mL serum-containing medium for collection.

4b. Keep on ice and run on sorter.

5. Spin down collection tubes and resuspend for plating.

Functional assays

1. Resazurin cytotoxicity assay
2. Seed 2000 cells/well in 96-well plate, with at least 8 dilutions/treatment and 3 replicates/dilution.
3. After 48 h, prepare treatment dilutions, including untreated (0).
4. Aspirate media from each dilution and add treatment, going from lowest to highest concentration.
5. After 72 h, add 10% resazurin per well (20 μL in 200 μL).
6. After 16 to 20 h, insert into plate reader, open SpectraMax software and measure

- fluorescence at

- absorbance at 560 and 600 nm

1. Copy data to excel, save as tsv and analyse dose-response curves in R.

1. LDH cytotoxicity assay
2. Competition assay

**Pre-exposure**

1. Culture hBECs on desired substrate (e.g. 60k cells/well in 6-well plates), half of the wells will be treated, half will not.

2. After 48 h, treat with 10% CSE (cigarette smoke extract) for 5 days.

**Staining & re-seeding**

3. Trypsinize and resuspend the cells in 1 mL FMED. Count and stain the pre-exposed and unexposed cells with 5 μL DiD and DiI, respectively.

**Re-exposure**

4. Wash with PBS and seed equal numbers of cells in direct co-culture (e.g. 10k+10k/well in 24-well plates).

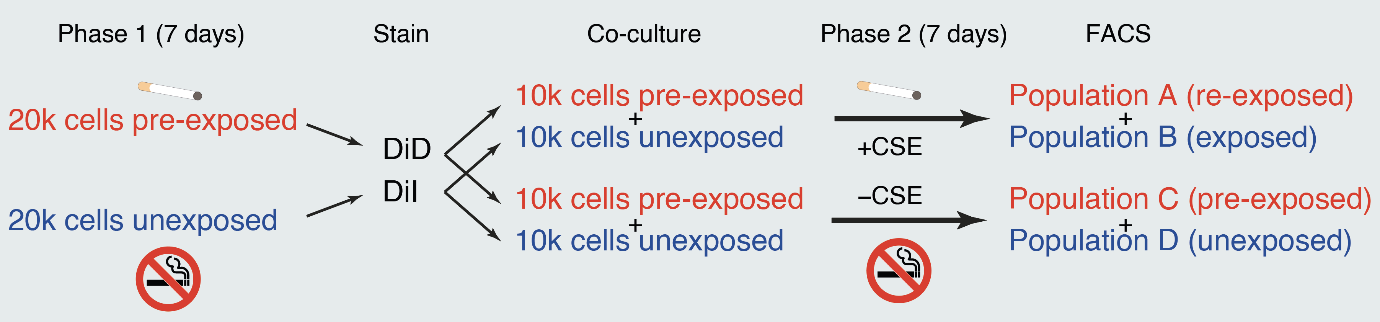
5. After 48 h, treat with 10% CSE for 5 days. If possible, re-add CSE 3 days later.

**Analysis**

6. Samples can be imaged on epifluorescence or confocal microscope.

7. Trypsinize, wash with PBS and stain with DAPI as viability dye. Can also stain for other antigens with the appropriate compensation controls.

8. Analyse on Fortessa or sort.



1. Colony formation assay
2. Coat 6 well plate with collagen for 30-60 min and air dry plates inverted.
3. Mitomycin feeders for 3 h and seed in coated 6 well plate (190k cells/well)
4. The next day, culture 1k HBECs for 10 days, changing medium every 3 days.
5. Fix with 500 μL PFA/well for 10 minutes, then add 200 μL crystal violet/well for 10 min.
6. Wash with PBS three times.
7. Air dry plates inverted.
8. Sphere formation assay
9. 2D migration (scratch) assay
10. 3D invasion (transwell) assay
11. GSH/GSSG assay

**Sample preparation**

0. Prepare 1x assay buffer by diluting 1:1 with ddH2O.

1. Wash 1 million cells in cold PBS.

2. Centrifuge at 300 G for 5 min at room temperature.

3. Remove PBS and lyse the cell pellet by homogenization or sonication in 200 μL of **cold buffer containing 50 mM phosphate, pH 7, 1 mM EDTA, and 20 μL of Scavenger**. (NO SCAVENGER FOR TOTAL GSH) (VOLUMES MUST NOT BE CHANGED)

4. Centrifuge at 10,000 G for 10 minutes at 4 C.

5. Transfer the supernatant to a clean tube and proceed to the deproteination procedure.

**Deproteination**

6. Prepare a **meta-phosphoric acid solution** in purified water at 5 grams per 100 mL. This reagent must be prepared fresh daily.

7. Add 65 μL of the MPA solution to 25 μL of sample. Briefly vortex to mix and then centrifuge at 14,000 G for 5 minutes.

8. Mix 6 μL of clear supernatant with 244 μL of Assay Buffer in a new tube.

9. Add 200 μL of each sample to a clear 96-well plate.

**Glutathione standard preparation**

10. Prepare a 300 μM GSH Standard by mixing 1 μL of the 100 mM GSH Standard with 332 μL of purified water.

11. Prepare a 3 μM GSH Standard by mixing 5 μL of the 300 μM GSH Standard with 495 μL of Assay Buffer.

12. Prepare five 200 μL GSH standards directly on plate, making up with assay buffer: 100%, 50%, 25%, 12.5% and 0%.

**Working reagent**

13. Mix enough reagent for the number of assays to be performed. For each Standard and Sample well, prepare 106.75 μL of Working Reagent: Assay Buffer 105 μL + GR Enzyme 1 μL + NADPH 0.25 μL + DTNB 0.5 μL.

14. Mix the Working Reagent *immediately* after adding the DTNB reagent.

**Measurement**

15. Add 100 μL of the Working Reagent to each Sample and Standard well. Tap plate to mix.

16. Read the optical density (OD) of each well at 412 nm at zero and 10 minutes (OD0Min and OD10Min respectively)

Total GSH levels were analyzed using the Glutathione Assay Kit (Cayman Chemical, catalog no. 703002).

U-2 OS cells were seeded at a density of 100,000 cells per well in McCoy’s 5A medium in six-well plates.

Following treatment, cells were washed once in ice cold PBS.

DMSO-treated cells were scraped into 500 μL of MES collection buffer while Era2-treated cells were scraped into 50 μl to concentrate depleted GSH due to low assay sensitivity.

GSH measurements were normalized to protein concentration that was equivalently concentrated with this method.

Cells were sonicated with ten 1-s pulses at maximum amplitude with a Fisher Scientific Model 120 Sonic Dismembrator (Thermo Fisher) to lyse cells.

To exclude debris, lysate was centrifuged at 12700 rpm for 15 min at 4 °C and supernatant was isolated.

Then, 25 μl of lysate was aliquoted and stored frozen at −20 °C for under 1 week before a Bradford Assay was performed to determine the protein concentration for normalization.

The rest of the lysate was deproteinated by adding one volume of 0.1 g ml−1 metaphosphoric acid (Acros Organics), vortexing thoroughly, then centrifuging at 12700 rpm for 3 min at room temperature.

Metabolite extract was stored at −20 °C for under 1 week before total GSH and glutathione disulfide (GSSG) levels were determined using Ellman’s reagent (DTNB, 5,5′-dithio-bis-2-nitrobenzoic acid) according to the manufacturer’s instructions.

Data are presented as DNTB fluorescence normalized to total protein or to a known GSH standard curve.

1. UGT1A10 assay

1. **2.6.1. β-Estradiol glucuronidation assay**

UGT activity of β-estradiol was assessed using the method described by [Kakehi et al. (2015)](https://www.sciencedirect.com/science/article/pii/S1532045618302060" \l "bb0105) with slight modifications. First, hepatic microsomal solution was mixed with KPB (0.1 M, pH 7.4) and prepared to 4 mg/ml. Aliquots of microsomal preparations (12.5 μl) were then mixed with 35 μl of KPB and 2.5 μl of 1% sodium cholate solution, and incubated on ice for 30 min. Then, 50 μl of microsomal solution was mixed with 41.5 μl of KPB (0.1 M, pH 7.4), 5 μl of 100 mM MgCl, and β-estradiol, which was dissolved in methanol, resulting in a final concentration of 1% methanol in a total volume of 97.5 μl. The final β-estradiol concentration was varied between 12.5 μM and 500 μM. Samples were then preincubated in a water bath for 5 min. The reaction was initiated by adding 2.5 μl of 100 mM UDPGA. After incubation for 15 min, the reaction was stopped by adding 200 μl of ice-cold methanol. The temperatures for preincubation and reaction were 41.5 °C for turkey, 42 °C for chicken, 38.5 °C for canary and ostrich, 40.5 °C for white-tailed eagle, 39.5 °C for great horned owl, 39.0 °C for Humboldt penguin, and 37.0 °C for rat ([McNab and Brian, 1966](https://www.sciencedirect.com/science/article/pii/S1532045618302060" \l "bb0135); [Siegfried et al., 1975](https://www.sciencedirect.com/science/article/pii/S1532045618302060" \l "bb0195); [Chaplin et al., 1984](https://www.sciencedirect.com/science/article/pii/S1532045618302060" \l "bb0035); [Herrero and Barja, 1998](https://www.sciencedirect.com/science/article/pii/S1532045618302060" \l "bb0085); [Richards, 1971](https://www.sciencedirect.com/science/article/pii/S1532045618302060" \l "bb0175)). Reaction mixtures were then placed on ice for 15 min before centrifugation at 15000 ×*g* for 10 min. The resultant supernatants were injected into a liquid chromatography/mass spectrometry (LC/MS) system. The HPLC system (pump: LC-20 CE; auto sampler: SIL-20A; column oven: CTO-20A; controller: CEM-20A; Shimadzu) coupled with [electrospray ionization](https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/electrospray-ionization) triple quadrupole mass spectrometry (ESI-MS/MS) (LCMS-8040; Shimadzu) was equipped with an Inertsil ODS-3 column (2.1 mm × 150 mm, particle size 5 μm; GL Sciences, Inc., Tokyo, Japan). The collision energies (CE) and other MS parameters were optimized and are shown in Supplementary Table S2. Mobile phase A consisted of 10 mM ammonium acetate buffer (pH 5.0) and phase B consisted of phase A:acetonitrile (1:9 v/v). The solvent gradient was as follows: 5% mobile phase B from 1.5 to 7 min followed by a linear gradient to 95%, 95% mobile phase B from 7 to 8.5 min, and then 5% mobile phase B from 8.5 to 10 min. An injection volume of 5 μl, flow rate of 0.3 ml/min, and column temperature of 45 °C were used.

https://www.sciencedirect.com/science/article/pii/S1532045618302060#bb0105

Animal experiments

1. Oral gavage
2. Prepare tamoxifen solution, 20 mg/mL in 10% ethanol in corn oil (v/v). First warm ethanol up to 55 °C and add to tamoxifen powder, then mix with corn oil, vortex and dissolve at 37 °C for 1 h.
3. Prepare sugar in water solution, filter and freeze aliquots.
4. Bring tube with tamoxifen, tube with sugar and gavage needles.
5. Prepare damp chow.
6. Place mouse on balance and note down weight.
7. Prepare syringe with 200 μg of tamoxifen/g of body weight (e.g. 200 μL for 20 g mouse).
8. Scruff mouse (left hand) making sure the neck is tight enough to prevent movement of the head or mouth, but not too tight, to prevent strangulation.
9. Insert needle into mouth at an angle and push back a bit once in the throat to enter the esophagus.
10. Once inside, insert into stomach and release tamoxifen.
11. Remove syringe and gently put mouse back into cage.
12. Add damp chow to cages.
13. Oropharyngeal delivery
14. Prepare BaP solution, bring vials for each concentration in a heating block at 60C in a polystyrene box.
15. Prepare stand, 200 μL pipette, 200 μL cut tips and two blunt tweezers.
16. Prepare isoflurane chamber, put paper towel inside. Make sure knob on iso tank is turned all the way to the right, flow knob is directed towards chamber and tube adaptor behind chamber is closed. Run scavenger at max (5 L/min).
17. Place mouse on balance and note down weight.
18. Place mouse in isoflurane chamber and turn on the machine with a flow rate of 2 L/min and 5% isofluorane. After 1 min (when mice are asleep), lower the iso to 2.5%. After another 2 min, take first mouse.
19. Place the mouse on holder so that mouth is kept open by metal wire or elastic band.
20. Take tongue out with tweezers.
21. Pipette substance up to 50 μL into mouth (15 μL of 2% (w/v) polidocanol, 40 μL BaP, etc.).
22. Pinch nose (pull skin around nose with tweezers). Mouse will switch to mouth breathing and make a popping sound. Let it breath in 3 times.
23. Gently put mouse into cage, turn iso to 0 so that chamber is empty for next mouse
24. Repeat with all mice.
25. Intraperitoneal delivery & organ harvesting
26. Prepare 50 mL Falcons with **PBS+0.05% ProClin 300** and **fixative (4% PFA or PAXgene)**. Prepare eppendorf with working solution of **pentobarbital (Dolethal)** by diluting 1:1 with **saline**.
27. Scruff mouse (left hand) making sure belly skin is tight and tail is under control.
28. Inject 100 μL (**5 μL/g**) of dilute pentobarbital (27G) into belly halfway between hip and midline, upwards at a 20 degree angle and towards midline.
29. Once the mouse is no longer responsive to leg pinch, immobilise on cork board with 23G needles or tape on hands and feet.
30. Cut skin/fat below ribcage as a triangle towards both shoulders, cut diaphragmatic pleura and skin all the way left and right. Cut sides of ribcage and pull back, holding it with the weight of scissors/haemostat.
31. Optional: harvest immune-related organs (spleen, thymus, lymph nodes).
32. Carefully remove pericardial sac.
33. Optional: prepare a **1.5 mL eppendorf with 50 μL of 50 mM EDTA** and wash inside of **29G insulin syringe** with it. Then collect 300-500 μL of blood by piercing the bottom of the right ventricle (black spot) with a 29G insulin syringe.
34. **CONFIRMATION**: pierce right atrium with curved scissors. Use paper towel to absorb blood.
35. Perfuse heart with **21G needle and 20 mL syringe with PBS** by injecting into the right ventricle (whole body perfusion) or left ventricle (same spot as blood collection, pulmonary perfusion) until lungs become white and somewhat inflated.
36. Cut sternum with scissors aimed upwards all the way to the larynx, exposing the trachea beneath.
37. Pull **thread** under upper trachea and tie square knot around it loosely.
38. Make small hole on frontal upper trachea and insert **butterfly cannula** connected to **tubing** via a **Discofix luer lock**. Tighten knot after inserting cannula. The tubing is connected to a **20 mL syringe taped** to the wall 12 cm above the bench. Approx. 5 mL PFA to be used per mouse for lung fixing.
39. Optional: for cell dissociation, use 5 mL syringe with 2 mL dispase directly linked to butterfly cannula. If any lobes/lungs are to be fixed, they should be removed prior and injected directly with fixative. The lungs can then be tied with thread at the required location to prevent dispase leaking.
40. Turn luer lock and run PFA until all lobes are inflated.
41. Remove cannula and tie knot tighter and into a double square knot *immediately*.
42. Cut over trachea and remove skin/ribs to the side, then from the top of the mouse cut under trachea and lungs and separate from other organs. Place them in falcon tubes with fixative or PBS.
43. Put cadaver in red bag in freezer.
44. Wholemount immunostaining
45. Fix tissues with 4% PFA for 2 h in ice.
46. Wash twice with PBS. Tissues can be stored in 0.05% ProClin/PBS at 4°C at this stage.
47. Microdissect tissues to expose airways or region of interest. Transfer to an appropriate container for staining (microcentrifuge tube or multi-well plate, depending on the tissue).
48. Permeabilise with 0.5% Triton X/PBS for 2h at RT or O/N at 4°C with gentle rocking.
49. Block in 5% serum/4%DMSO/2.5%BSA/0.5% Triton/0.05% ProClin/PBS for 2-3h at RT or overnight at 4°C, with gentle rocking

\*Serum should be from same species as secondary antibodies.

1. Incubate with primary antibodies in blocking solution for 48h at 4°C with gentle rocking.
2. Wash 3 x 2h in 0.1% Triton X/PBS (PBST) at RT with gentle rocking.
3. Incubate with secondary antibodies (1:100 if using AF405, 1:200 for Jackson Immunoresearch abs and 1:500 for Lifetech abs) in blocking solution for 48h at 4°C protected from the light and with gentle rocking.
4. Wash 3 x 2 h in PBST at RT protected from the light and with gentle rocking.
5. *Optional:* Stain nuclei with 0.1 μg/mL DAPI in PBST for 30 min with gentle rocking. Wash 2 x 1h in PBST with gentle rocking. Protect from light.
6. Wash 1 x 1 h with 0.05% ProClin/PBS with gentle rocking. Tissues can be stored at this stage at 4°C.
7. Transfer the tissue onto a petri dish and check under the fluorescence microscope for any fibres or particles displaying autofluorescence in the DAPI channel. Carefully remove them under the dissection microscope. Cut trachea open after this. Bring RapiClear 1.52 (SunJin Labs) to RT during this step.
8. Fix spacer (iSpacer, SunJin Labs) of appropriate thickness onto a clean coverslip using the double-sided stickers provided. Add RapiClear in the centre (volume depends in thickness and size of the spacer) covering the area where the tissue will be placed. Avoid making bubbles. Place tissue inside the spacer with the surface of interest facing the coverslip, cover with RapiClear and carefully adhere a coverslip onto the spacer, press gently. Seal edges with nail polish.
9. Store slides flat, in the dark at 4°C.