## Genotyping of mice by LacZ staining

Earpunch mice into PBS in a 96-well plate (50 ul per well). 50 ʎ PBS

Fix 5-30 minutes (15 minutes to 1 hour) in 0.2% glutaraldehyde in PBS. 50 ʎ Fix
(Shortcut: add equal volume of 0.4% glutaraldehyde in PBS)
Wash 5 minutes (or more) in PBS 1x or 2x. 100 ʎ
Stain with X-gal stain (see below; may reuse old stain) at 37°C (or at RT), 100 ʎ
protecting from light. *Staining is usually visible after 5 minutes if fresh stain used.

### LacZ Staining

**LacZ Fix:** (for 50 mL)
- 0.4 mL 25% glutaraldehyde — 8 ml
- 1.0 mL 250 mM EGTA pH 7.3 — 1 ml
- 0.1 mL 1 M $MgCl_2$ — 1
- 100 mM sodium phosphate, pH 7.3 or PBS to 50 mL

0,4

**Wash Buffer:** (for 500 ml)
- 1.0 mL 1 M $MgCl_2$
- 1.0 mL 1% sodium deoxycholate (NaDC; make up in water; store in fridge)
- 5.0 mL Nonidet-P40 (make up in water; store in fridge)
- 100 mM sodium phosphate (pH 7.3) or PBS to 500 mL

**X-gal Stain:** (for 50 ml)
- 2.0mL 25 mg/mL X-gal dissolved in dimethyl formamide (stored in freezer)
- 1.0 mL of 250 mM potassium ferrocyanide + 250 mM potassium ferricyanide
  (Stored light-protected at room temperature)
- 47.0 mL Wash Buffer

1) ears in PBS
2) suck away PBS, add 100 ʎ fix
3) fix ~ 10min
4) suck away fix, add 100 ʎ PBS, wash ~ 15min
5) suck away PBS, add stain, cover with Alu,
let sit ON in 37°C

## LacZ staining protocol (May 2000)

Lac Z staining of postimplantation embryos, tissues and cells (Nagy lab).

### 1. 0.1 M phosphate buffer pH 7.3

115 ml of 0.1 M Sodium Phosphate monobasic (MW 155.99) (1.8 g per 500 ml)
385 ml of 0.1 M Sodium Phosphate dibasic (MW 268.07) (10.32 g per 500 ml)

This mixture should give a pH of 7.3 Alternatively:

141 g $Na_2HPO_4$ + 8 ml 85% phosphoric acid per 2 L (pH 7.3 to 8.5) – try pH 8.0

> A background staining is seen in yolk sac of day 10 embryos and a thin stripe of staining is observed in hindbrain of day 12 embryos. Increasing pH of Phosphate buffer might help to reduce background. The procedure works at pH 8.5.

### 2. Lac Z fixative solution

Might be prepared ahead except for the *gluteraldehyde* (*added fresh each time*)

| | |
|---|---|
| 0.4 ml | 25 % gluteraldehyde (Sigma G6257) |
| 2.5 ml | 100 mM EGTA (pH 7.3) MW 380.4 (3.804 g per 100 ml) |
| (1.0.ml | 250 mM EGTA (pH 7.3)) |
| 0.1 ml | 1M $MgCl_2$ |
| | 0.1 M Phosphate Buffer pH 7.3 or PBS up to |
| 50.0 ml | |

### 3. Wash buffer

| | |
|---|---|
| 0.4 ml | 1M $MgCl_2$ |
| 2.0 ml | 1% Sodium deoxycholate (NaDC; make up in water, store at 4 °C) |
| 2.0 ml | 2 % Nonidet-P40 (make up in water, store at 4 °C) |
| 195.6 ml | 0.1 M Phosphate Buffer pH 7.3 or PBS |
| 200.0 ml | |

### 4. X-gal stain

**Final concentration**
1 mg/ml X- gal
2 mM MgCl2
5 mM K4Fe(CN)6 x 3H2O
5 mM K3Fe(CN)6

in wash buffer or PBS without Ca and Mg

100 X solution of potassium ferrocyanide and ferricyanide might be prepared ahead and stored foil-covered at room temperature. X-gal should be added fresh each time.

| | |
|---|---|
| 2.0 ml | 25mg/ml X-gal stock solution in di-methyl-formamide (stored in the dark, at –70°C or –20°C) |
| 0.106 g | Potassium Ferrocyanide (Sigma P9387) |
| 0.082 g | Potassium Ferricyanide (Sigma P8131) |
| (1 ml | 250 mM Potassium ferrocyanide + 250 mM Potassium Ferricyanide stored light-protected at room temperature) |
| | Wash buffer up to |
| 50.0 ml | |

Alternatively:

| | |
|---|---|
| 48.0 ml | PBS (without Ca and Mg) + 2 mM $MgCl_2$ (deoxycholate and Nonidet-P40 can be omitted in the wash buffer to increase the intensity of staining) |
| 50.0 ml | |

After staining, solution can be reused following filtration through Whatman paper and stored at -20°C in the dark.)

**LacZ staining of postimplantation stage embryos**

1. Dissect embryos in PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ in Petri dishes under a dissection microscope.
2. Transfer of embryos is achieved using a 1ml pipetman and a blue tip with the end cut off or Samco Transfer pipettes (Cat. # 222-20S). Embryos are fragile and will be damaged if transferred with forceps.
3. Rinse embryos in 0.1 M Phosphate buffer at room temperature (optional).
4. Fix embryos at room temperature with optional mixing for 30 to 90 minutes depending on the size of the embryo. (For example day 9.5 embryos are fixed for 60 minutes or 30 min on ice with shaking).
5. Wash embryos three times for 15-30 minutes each at room temperature in wash buffer.
6. Stain embryos in the dark with mixing for 2 hours to overnight depending on a level of Lac Z activity. Staining is usually performed at 37°C, but if done at

7. Stain solution is replaced with wash buffer and samples are stored at 4°C (staining will intensify in wash buffer with time). Embryos can also be fixed after staining in fresh solution of 4% formaldehyde (Fisher F 79-1) in PBS.

For samples to be cryosectioned after washing cryoprotect in 15% sucrose in PBS for 1 hour at 4 ° C, then in 30% sucrose in PBS overnight at 4 °C. Incubate in O.C.T. at 4 °C for 1 hour or more prior to embedding in O.C.T. over dry ice.

Alternatively, for day 12.5 embryos and older as well as for adult tissues the samples might be sectioned sagittally after 30 minutes in pre-fix solution (prepared fresh): 2% Paraformaldehyde in PBS or 2% formaldehyde in PBS added to the regular 0.2% Gluteraldehyde fixative.

1. Embryos are kept in fix for 30 minutes at room temperature (with shaking). Larger organs are fixed for 1 hour.
2. Cut embryo sagitally using a new razor blade
3. Fix an additional 30 minutes to 1 hour on ice.
4. Replace the fixative with fresh LacZ fix (gluteraldehyde), keep for additional hour.
5. Wash x3 for 15-30 minutes in wash buffer (samples to be cryosectioned could be washed in PBS).
6. Proceed with LacZ stain at 37° C or room temperature for 30 minutes to overnight, protecting from light, best if done with shaking. Cryoprotect samples to be cryosectioned.

**Lac Z staining of cells**

1. Rinse cells with PBS x2
2. Add fresh fix (160 ul of 25% Gluteraldehyde in 20 ml of PBS). Fix for 5- 10 minutes at room temperature.
3. Wash with PBS x3
4. Add X-gal stain, protect from light, incubate at RT or 37 C.

**Genotyping of mice by Lac Z staining of ear punches**

Earpunch mice into PBS in a 96-well plate (50 ul per well).
Fix 15 minutes to 1 hour in 0.2% gluteraldehyde in PBS.
(Shortcut: add equal volume of 0.4% gluteraldehyde in PBS)

0.4 % Gluteraldehyde – 2x stock:

| | |
|---|---|
| 80 ul | 25% gluteraldehyde |
| 5 ml | PBS |

Wash 5 minutes (or more) in PBS 1x or 2x. Stain with X-gal stain (see below; may reuse old stain) at 37°C (or at RT), protecting from light. *Staining is usually visible after several hours at 37 C, can be left O/N at room temperature.

transfer the cell suspension into the new V-bottom 96-well plate, containing 2x freezing media and kept on ice.

- Add 50µl of cold sterile mineral oil (e.g. Sigma M8410, or Specialty Media #ES-005-C) on top of each well.
- Wrap the plates in parafilm and foil (the latter is optional). Place in a pre-cooled styrofoam box, and store in a –70°C freezer, preferably not longer than two months, until ready for thawing and expansion.

The first frozen plates are considered as master plates. The unfrozen replica plates are used for further characterization of the clones. After strong expresser and single copy integrant clones have been identified, the frozen stock can be thawed and expanded for further analysis.

- Prepare the necessary number of 4- or 24-well feeder plates, containing ES-DMEM.
- Remove the plate containing identified clones from the freezer. Unwrap the plate and warm quickly (place in the incubator).
- When ice crystals almost disappear, wipe the outside of the plate with 70% ethanol.
- Add 100 µl of warm ES-DMEM to the wells under the oil and transfer the content of the wells into wells of newly prepared plates.
- Rinse the original wells of the 96-well plate with more ES-DMEM and transfer to the same wells. Change media after overnight culture and daily.
- Passage the cells when they reach 70-80 % confluency to a larger plate (i.e. 35 mm). If cells do not reach confluency in a few days but form few colonies in a well, they can be trypsinized, broken into smaller cell clumps and plated on the same plate.
- Passage 1:5 every other day, freeze the cells in vials as described.

### *DNA isolation from ES clones in 96-well plates*

#### Reagents

- Lysis buffer: 10 mM Tris-HCl, pH 7.5; 10 mM EDTA; 10 mM NaCl; 0.5% sarcosyl; 1 mg/ml Proteinase K added before use.
- NaCl/Ethanol mix: 150 µl of 5 M NaCl per 10 ml of cold 100% ethanol (prepared fresh).
- Restriction digestion mix (per well): 1x appropriate restriction buffer, 1mM spermidine, 100 µg/ml BSA, 50-100 µg/ml RNase, 10-20 units of enzyme. Use 35-40 µl per sample.

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# X-gal staining

1. Wash cells twice with PBS
2. Fix the cells by adding 5 mL of glutaraldehyde and incubating for 15 min
3. Remove the fix solution and rinse 3 times with PBS
4. Add 3 mL of X-gal solution to the cells. Incubate the cells at 37°C for 1-16 hours
5. Remove the X-gal solution, cover the cells with 1x PBS
6. For long term storage, store the cells under 70% glycerol at 4°C

**BUFFERS:**

**Fix solution:** 0.25% (v/v) glutaraldehye in PBS

**X-gal solution in PBS:**

0.2% X-gal (from 20 mg/mL (2%) stock in N,N-Dimethylformamide*)
2 mM $MgCl_2$
5 mM $K_4Fe(CN)_6 \cdot 3H_2O$
5 mM $K_3Fe(CN)_6$

| | 10 mL | 50 mL |
|---|---|---|
| 2% X-gal | 1 mL | 5 mL |
| 1M $MgCl_2$ | 20 μL | 100 μL |
| 100 mM $K_4Fe(CN)_6 \cdot 3H_2O$ | 500 μL | 2.5 mL |
| 100 mM $K_3Fe(CN)_6$ | 500 μL | 2.5 mL |
| | Top with PBS | Top with PBS |

* N,N-Dimethylformamide (DMF) from BDH, ACS297-76

# Lac Z staining - solutions.

* Lac Z stain ( 10ml )

- 400 µl X-gal
- 100 µl potassium ferrocyanide
- 100 µl " ferricyanide
- 9.4 ml wash buffer

10 ml lac Z solution

* Lac Z fix (for 1 ml)

- 8 µl glutaraldehyde in 1 ml of fix buffer.

* Making lac Z fix buffer:
0.4 ml of 25% glutaraldehyde
1.0 ml of 250 mM EGTA, pH 7.3 -
0.1 ml of 1M $MgCl_2$
add 100 mM sodium phosphate pH 7.
or PBS to 50 mL

make - 500 ml
i.e. x10

* Making X-gal stain: 2 mL 25 mg/ml X-gal dissolved in
dimethyl formamide (freeze
1 mL 250 mM potassium ferrocyanide
+250 mM " ferricyanide
47 ml wash buffer.

50 ml → 1
10 ml → 0.2 ml

* Making wash buffer (500 ml)

1 ml of 1M $MgCl_2$
1 ml of 1% NaDC (in fridge)
5 ml of Nonidet p-40 (in fridge) NP-40
100 ml of Sodium phosphate, pH 7.3 or
or PBS to 500 ml.

X-gal stain - 2 mL 25 mg/mL X-gal dissolved in
dimethyl formamide (in freezer)

- 1 mL of 250 mM potassium ferrocyanide
  + 250 mM potassium ferricyanide

+ 47 mL wash buffer

## Sexing of mouse embryos by PCR.

Small fragments of embryonic material can be used to sex postimplantation mouse embryos by duplex PCR specific for the X chromosome (*Xist* gene) and the Y chromosome (*Zfy1* gene). The following protocol can be used on small volumes (approx. 2 µl) of embryonic samples or yolk sac. For smaller samples (e.g., preimplantation embryos), the sensitivity is increased by incorporation of a $^{32}P$-labeled nucleotide during the reaction and detection by autoradiography instead of the standard ethidium bromide staining technique.

### 1. Sample

Collect the tissue sample in a 0.5 ml tube and wash with 20 µl of $dH_2O$.
Spin briefly and remove water.
Add 20 µl of lysis buffer (0.05% SDS, 0.035 M NaOH).
Heat 3 min @ 98°C in a PCR machine. Cool on ice.
Spin briefly and add 1 µl of 1.0 M Tris pH 8.3 to neutralise.

### 2. PCR analysis

Each PCR reaction is performed in a final volume of 20 µl, using 2 µl of sample. This amount may vary depending on the type and size of the tissue sample. Each PCR reaction contains:

| initial | vol. | final |
|---|---|---|
| sample | 2 µl | |
| 10xPCR buffer | 2 µl | 1x |
| 2.5 mM dNTPs | 0.8 µl | 100 µM |
| primers, 10 pmol/µl | 4 x 1 µl | 10 pmol |
| *Taq* polymerase, 5U/µl | 0.2 µl | 1 unit |
| $dH_2O$ | to 20 µl | |

Two primer pairs are used to simultaneously amplify a control fragment of the X chromosome (*Xist* gene, exon 1, 207 bp) and a region of the Y chromosome (*Zfy1* gene, 183 bp).

| | |
|---|---|
| X1F (20-mer) | 5'-TTGCGGGATTCGCCTTGATT-3' |
| X1R (20-mer) | 5'-TGAGCAGCCCTTAAAGCCAC-3' |
| ZFY1a (26-mer) | 5'-GACTAGACATGTCTTAACATCTGTCC-3' |
| ZFY1b (25-mer) | 5'-CCTATTGCATGGACAGCAGCTTATG-3' |

The PCR program performs 4 cycles with longer denaturation time (4 min), followed by 31 cycles with short denaturation steps of 1 min. The total number of cycles may have to be adjusted for different applications.

1. 95°C / 4 min
2. 65°C / 1 min
3. 72°C / 1 min
4. 3 times to step 1
5. 95°C / 1 min
6. 65°C / 1 min
7. 72°C / 1 min
8. 30 times to step 5
9. 72°C / 5 min
10. 4°C / hold

lacZ stain (for 10 mL)
400 μL x-gal (in fridge)
100 μL Ferro
100 μL Ferri
+wash buffer
10 mL solution

Protocol for lacZ staining —

1. put ears/tails in PBS.
2. remove PBS; add 100 μL of lacZ fix
3. fix ~ 10 min.
4. remove fix; add 100 μL of PBS
5. wash ~ 15 min
6. remove PBS; add 100 μL of X-gal stain, cover with parafilm & aluminum foil
7. Let sit overnight @ 37°C.

LacZ Fix (for 1 mL)
~~400 μL~~ (in fridge)
8 μL glutaraldehyde
in 1 mL of fix buffer.

→ making LacZ fix (50 mL): 0.4 mL of 25% glutaraldehyde
1.0 mL of 250 mM EGTA, pH 7.3
0.1 mL of 1M $MgCl_2$.
– add 100 mM sodium phosphate, pH 7.3 or PBS to 50 mL.

→ making wash buffer (500 mL): 1 mL of 1M $MgCl_2$.
1 mL of 1% NaDC (in fridge).
5 mL of Nonidet-P40 (in fridge)
100 mM of sodium phosphate, pH 7.3 or PBS to 500 mL.