SANGER INSTITUTE
STANDARD OPERATING PROCEDURE

SUBJECT: Collection and LacZ staining of embryos (wholemount and fixed frozen cryosections)

INTRODUCTION:

This document outlines the general procedures involved in the collection and staining of mouse embryos (wholemount or fixed frozen cryosections), for gene expression analysis of a LacZ reporter insert.

Litters were collected from either HET x HET matings (when obtained as part of a recessive lethality study), or from HOM/HET/HEMI (male) x WT (female) matings to obtain two heterozygous LacZ stained embryos per staining method.

Whole litters were processed as either wholemount LacZ stained or fixed frozen for cryosections. Upon genotyping, embryos could be selected for imaging (wholemount) or cryosectioning and staining (fixed frozen) as appropriate.

Sanger Recessive Lethality Pipeline = 1 litter of ED14.5 was wholemount LacZ stained
BASH LacZ Expression Pipeline = 1 litter of ED12.5 was wholemount LacZ stained, 2 litters of ED12.5 were fixed frozen for LacZ stained cryosections.

ABBREVIATIONS:

DCF = Data Capture Form
DMF = Dimethylformamide
GM = Genetically Modified
LAA = Laboratory Animal Allergens
NVS = Named Veterinary Surgeon
PBS = Phosphate buffered Saline
PFA = Paraformaldehyde
PIL = Procedure Individual Licence
PPE = Personal Protective Equipment
PPL = Procedure Project Licence
QC = Quality Control
RSF = Research Support Facility
SOP = Standard Operating Procedure

QUALITY CONTROL (QC):

1. Tissues should be analysed and imaged for LacZ staining as soon as possible after collection before stain diffusion occurs.
2. Positive control slides should be included in all LacZ staining experiments.
3. Cryosections from 2 heterozygotes, from different litters should be LacZ stained on the same day to minimize staining variation.

Problem / Issue	QC action
Failed Genotype	QC fail the data capture form (DCF).
Genotype and Staining pattern are inconsistent	Submit genotype confirmation request with genotyping team.

	If the genotype is confirmed and still does not match the staining, QC fail the DCF.
Inconsistencies in the LacZ staining pattern between wholemount stained hets from the same colony	Check the positive control slide for unusual staining. Compare with data from LacZ stained cryosections from the same colony (if available). Check genotypes and consider submitting genotype confirmation request (consult line manager beforehand). QC fail the DCF and request a replacement litter (consult line manager beforehand). Verify that staining reagents are effective before using again – refresh if necessary.
Inconsistencies in the LacZ staining pattern between 2 cryosectioned hets from the same colony	Check the positive control slide for unusual staining. Compare with data from LacZ stained wholemount samples from the same colony (if available). Check genotypes and consider submitting genotype confirmation request (consult line manager beforehand). Select an additional heterozygote frozen sample to section and stain. Verify that staining reagents are effective before using again – refresh if necessary.
LacZ staining present in 2 cryosectioned hets that is not apparent in wholemount stained hets from the same colony	Check genotypes and consider submitting genotype confirmation request (consult line manager beforehand). Consider strength of LacZ staining pattern. The stain may not penetrate wholemount samples very deeply and weak/ diffuse staining may be difficult to detect with a dissecting microscope.
LacZ staining present in wholemount stained hets that is not apparent in 2 cryosectioned hets from the same colony	Check genotypes and consider submitting genotype confirmation request (consult line manager beforehand). Select an additional heterozygote frozen sample to section and stain. Verify that staining reagents are effective before using again – refresh if necessary.
No LacZ staining apparent in any samples from the same litter	Check the positive control slide: If positive control slide is not stained QC fail the DCF and request a replacement litter. Verify that staining reagents are effective before using again – refresh if necessary.
No LacZ staining on positive control slide	Check the positive control slide: If no samples within the experiment are stained, QC fail the DCF and request a replacement litter. Verify that staining reagents are effective before using again – refresh if necessary.

HEALTH & SAFETY:

This procedure is covered by the following risk assessment: **WTSI_1209**

- Appropriate personal protective equipment (PPE) is to be worn at all times when handling animals. This includes overshoes, gown and gloves
- Entry procedure to the Research Support Facility (RSF) should be followed including the wearing of scrubs.
- **In addition to the above, when sources for laboratory animal allergens (LAA) (animals or soiled cages) are not contained within Local Exhaust Ventilation Systems (change stations, fume hoods or down flow tables), a respiratory mask, for which you have passed a face fit test, must be worn.**
- Access to a functional down-flow table and fume hood is required.
- Safety glasses must be worn when handling fixatives.

- Users should familiarize themselves with the location of emergency equipment including: Eye wash stations, fire extinguishers, and spill kits.
- This procedure can only be performed during core working hours.
- All electrical equipment is to be inspected for damage before use.
- Lone worker alarms should be used when working alone in the RSF.
- New workers are to be supervised until deemed competent to perform this assay.
- Individual risk assessments for young persons and new or expectant mothers would be performed to define any exclusion for performing this assay.

RESPONSIBILITIES:

All staff performing this procedure are responsible for ensuring that this Standard Operating Procedure (SOP) and accompanying risk assessment has been read and understood. This SOP should be followed in accordance with the relevant Procedure Project License (PPL).

All staff should be trained and competent to perform the procedure, where applicable they should also be licensed to perform the procedure with a valid Procedure Individual License (PIL).

RESOURCES:

Equipment and reagents:

1.	Fume hood and down flow bench	
2.	Polystyrene ice box & ice bucket	
3.	50ml falcon tubes	Sanger stores: PLTU0002
4.	Dissecting instruments	Fine Science Tools
5.	10cm and 6cm plastic petri dishes	Sanger stores: PLDI0018, PLDI0012
6.	7ml Bijoux	Sanger stores: PLBO0011
7.	Rocking platform shaker at 4°C	
8.	Genotyping PCR plates	ThermoFisher UK Ltd: MPP-114-101R
9.	PCR plate cooler blocks	
10.	Aluminium foil	
11.	Magnetic stirrer and stirrer bars	
12.	Cold PBS pH 8.0	Sanger Media team
13.	Sodium hydroxide (4 mol/l (4 N) in aqueous solution) to pH PBS	BDH (VWR) 191373M
	Sodium hydroxide causes damage to organs and is corrosive. To be used only under an effective fume hood or on a functional downflow table wearing correct PPE.	
14.	32% Paraformaldehyde fixative Dilute to 4% PFA with PBS pH 8.0	Electron Microscopy Sciences; Catalogue No. 15714-S
	PFA is toxic, harmful, corrosive, an irritant, a skin sensitizer, possibly carcinogenic and can cause damage to organs. To be used under an effective fume hood and wearing correct PPE.	
15.	50% Glycerol in PBS pH7.4	Sanger Media team
16.	70% Glycerol in PBS pH7.4	Sanger Media team
17.	Sodium Azide	Sigma: S8032 500g
	Sodium Azide is toxic and an irritant. To be used only under an effective fume hood wearing correct PPE	
18.	LacZ Staining Solution: (Appendix B)	

	- 2 mM MgCl2.6H2O	Sigma: Catalogue No. M9272
	- 0.01% Deoxycholic Acid	Sigma: Catalogue No. D2510
	- 0.02% IGEPAL CA-630	Sigma; Catalogue No. I8896
	- 5mM potassium ferrocyanide (C6FeK4N6.3H2O)	Fisher: Catalogue No. P/4920/53
	- 5mM potassium ferricyanide (C6FeK3N6)	VWR: Catalogue No. 26807.297)
	- 0.1% (1mg/ml) X-Gal in DMF	Invitrogen: Catalogue No. 15520018 Sigma: Catalogue No. D4551
	Dimethylformamide (DMF) is harmful, flammable, causes damage to organs and an irritant. To be used under an effective fume hood and wearing correct PPE. Use glass cylinders as can melt plastic.	
	- Cold freshly made PBS pH 8.0	Invitrogen: Catalogue No. 70011051
19.	Dissection and Wholemount Imaging: Microscope Computer Light box	 Leica MZ16A stereomicroscope 0.5x Planapochromatic objective (10446157) Leica LAS v4.4 software
20.	Imaging plates: - Deep 14cm diameter petri dish - Silicon: Sylgard 184 Silicone elastomer kit	 Sigma: Catalogue No. D9054-1CS VWR: 634165S
21.	Fixed frozen cryosection 30% sucrose w/v in PBS	VWR: 27480 Sucrose: (30g sucrose, make up to 100 mL)
22.	OCT	VWR International Ltd – No. 361603E
23.	Embedding moulds	VWR International Ltd. – Base mould 7x7 – No. 720-0823
24.	Dry Ice	Collected on site: BOC
25.	Isopentane	Sigma-Aldrich Co. Ltd – No.320404-1l
	Isopentane is fatal/very toxic, extremely flammable and causes damage to organs. To be used only under an effective fume hood wearing correct PPE.	
26.	Thermometer	VWR International Ltd – No 620-0860
27.	Metal (cold proof) container	Scientific Laboratory Supplies Ltd. – SLS7589
28.	Blotting paper	Scientific Laboratory Supplies Ltd. – No. MIC4070
29.	Hybridisation Oven	Techne Hybrigene
30.	Nuclear Fast Red counterstain	Vector Nuclear Fast Red H-3403 500ml
	Nuclear fast Red is an irritant and causes damages to organs. Wear appropriate PPE when handling	
31.	Xylene	Xylene mix of isomers AnalaR NORMAPUR 2.5L: VWR #28975.325
	Xylene is highly flammable, an irritant, causes damage to organs and is a carcinogen. To be used only under an effective fume hood wearing correct PPE.	

32.	Ethanol absolute	Ethanol absolute AnalaR NORMAPUR: VWR 20821.321
	Ethanol is highly flammable. To be used only under an effective fume hood wearing correct PPE.	
33.	Cryostat	Leica cryostat CM3050 S
34.	-80°C Freezer	Sanyo VIP series
35.	Slide storage boxes	Scientific Laboratory Supplies Ltd. – No.HS15991A
36.	Slide staining box	VWR International Ltd. – 631-0358
37.	Slide rack	VWR International Ltd. – No. 631-0357
38.	Troughs for staining station	Thermo Scientific A78010487
39.	Plastic Tupperware boxes	
40.	Ultra-pure sterile water	Sanger Media Team
41.	Automated coverslipper (optional) Coverslipper racks Coverslip cassette	Thermofisher Clearvue Coverslipper Thermo Scientific Cleavue Gemini Compatible baskets. Black # A79210064, White # A79210065 Thermo Scientific A78210051
	Charcoal filter Mountant	Thermo Scientific Shandon vapour filter #12623106 Thermo Scientific Clearvue mountant XYL 473ml bottles
	Mountant (for manual mounting)	Leica CV Mount 14046430011
	Mountant (contains 60% Xylene) – Xylene is an irritant, harmful, highly flammable, causes damage to organs and is a possible carcinogen. It must only be used in an effective fume hood and appropriate PPE must be worn.	
42.	Brightfield Slide scanner	Hamamatsu Nanozoomer 2.0HT NDP.scan v2.5 software.
43.	LacZ positive control slide Fixed frozen cryosections from adult mouse tissue known to stain positively for LacZ reporter.	

Staff:
One person is required to perform this procedure.

PROCEDURE A: Collection of Embryos in the RSF

Before performing the procedure, verify that this is the correct mouse for the procedure. A maximum of one litter per dissector should be collected at any one time.

1. Prepare and label 50 mL falcon tubes of cold PBS pH pH7.4 (one per litter collected) and place on ice.

   Note: Once removed from the mother the uterus should spend **no more than 30 minutes on ice.**

2. **On a down-flow table,** sacrifice the female by cervical dislocation. Check if the female is unresponsive. Damp the fur with 70% Ethanol, open up the body cavity and confirm death my removal of the heart.

3. Remove the uterus. Count the embryos (and resorptions as appropriate), record on mating card for database entry later.

4. Transfer uterus to cold PBS on ice and transport to lab.
   The residual animal carcass must be disposed of in a clinical waste bag, sealed, and put in the relevent freezer.

   Clean equipment and surfaces. All dissection instruments should be scrubbed clean of blood in the sink soaked in antibacterial solution.

PROCEDURE B: Embryo dissection in the Lab

1. Under a dissecting microscope, remove embryos from the uterus keeping the placenta and yolk sac intact. Recount the embryos (and resorptions as appropriate). Transfer embryos into individual petri dishes of clean PBS (to avoid contamination) and place on ice.

2. Extract embryos from the yolk sac and embryonic membrane. Separate the embryo from the placenta.

3. Collect two small pieces of yolk sac for genotyping, into two 96 well genotyping plates (on cooler block). Label plates Yolk 1 (for submission) and Yolk 2 (for back-up). Clean forceps with ethanol wipes between different embryo samples.

4. Check the gross morphology of the embryos. If the embryo appears abnormal, take an image to show the dsymorphology and record on the cage card for database entry later.

5. For wholemount stained ED12.5 embryos only: Pierce the back of the head in the fourth ventricle (Appendix A).

6. Using a perforated moria spoon transfer the embryo and placenta, into a labelled 7ml bijou containing ice cold PBS.

7. Let embryo bleed out for a while in PBS (haemoglobin inhibits the staining reaction) keeping the embryos on ice.

PBS waste containing biological contaminants much to treated with Virkon for 10 minutes, then diluted 5 times with water and disposed of down a laboratory sink.

Solid biological waste must be frozen in the -20C in a biohazard bag and given to the Sanger Waste Team for disposal.

Transfer all other waste to a yellow clinical waste bag or clearly labelled waste container.

Clean equipment and surfaces. All dissection instruments should be scrubbed clean of blood in the sink soaked in antibacterial solution.

Fixation

Note: Embryos should never be left dry. When changing solutions, always keep embryos in enough residual solution to cover them as they are extremely fragile and can easily dry out and become damaged.

8. **In a fume hood**, remove the PBS from the embryos and replace with 4% PFA pH8.0
   Note: 4% PFA loses its effectiveness with time. Use freshly diluted 4% PFA solution (from 32% PFA stock) and use within 1 week.

9. Fix on rocker at 4°C for 20 minutes.

10. **In a fume hood**, remove the PFA and replace with PBS pH8.0. Place on rocker at 4°C for 30 minutes. Repeat for a total of three 30 minute washes.

    Note: Overfixation with PFA will inhibit the beta-galactosidase involved in the LacZ staining reaction.

    PFA waste and washes must be disposed into the PFA waste container and given to the Sanger Waste Team for disposal.

LacZ staining of Wholemount embryos continues in
PROCEDURE C: Wholemount LacZ staining of fixed embryos
PROCEDURE D: Analysis and imaging of Wholemount LacZ stained embryos

LacZ staining of Fixed Frozen cryosections continues in
PROCEDURE E: Freezing of fixed embryos
PROCEDURE F: Cryosectioning of fixed frozen embryos
PROCEDURE G: LacZ staining of cryosections
PROCEDURE H: Analysis and imaging of LacZ stained slides

PROCEDURE C: Wholemount LacZ staining of fixed embryos (see Appendix B for stock reagents)

LacZ staining

1. **In a fume hood**, make up LacZ solution (excluding x-gal) in a glass bottle (6 ml per embryo + 10ml for positive control slide).
   Add the X-gal just before use. Ensure the LacZ solution is thoroughly stirred using a magnetic stirrer. (See Appendix B)

   (For details of positive control staining, see Appendix C)

Final Concentration	Volume of Stock reagent: 1 embryo + positive control	
2mM MgCl2.H2O	160	uL
0.02% IGEPAL	160	uL
5mM potassium ferrocyanide	800	uL
5mM potassium ferricyanide	800	uL
Cold PBS pH 8.0	14	mL
0.01% deoxycholic acid	16	uL
0.1% (1mg/ml) X-Gal in DMF	400	uL
Total	16 mL	

2. **In a fume hood**, pour off the PBS from samples and replace with LacZ staining solution. Incubate sampes in LacZ staining solution for up to 48hrs in the dark, on a rocker at 4°C.

Post-staining fixation and clearing

3. **In a fume hood**, remove the LacZ stain solution from wholemount samples and replace with 4% paraformaldehyde. Leave overnight on a rocker at 4°C.

4. **In a fume hood**, remove the PFA and replace with 50% glycerol. Leave to clear overnight on a rocker at 4°C.

5. Remove the 50% glycerol and replace with 70% glycerol. Leave to clear overnight on a rocker at 4°C. Cleared samples can be left at room temperature until imaged, store in the dark (closed cardboard box).

6. Image stained tissues within 3 months (LacZ stain has been found to diffuse away over time). For long term storage, transfer tissues to 70% glycerol with 0.01% sodium azide.

PROCEDURE D: Analysis and imaging of Wholemount LacZ stained embryos
(See Appendix D for standardised annotation list and representative tissue imaging)

1. Examine each embryo and placenta in a litter under a microscope for the presence or absence of LacZ staining. Record the staining result for each embryo and placenta on the DCF as “Present” or “Not detected”.

   If the staining pattern is typical of the endogenous staining seen in wild-type embryos, record it as “Not detected”. Record an “Ambiguous” staining result where the staining result is uncertain e.g. very faint, non-discrete staining but atypical for endogenous staining.

   For examples of endogenous LacZ staining in wild-type embryos, refer to lab WT image example catalogue and any wild-type embryos stained in the same litter.

2. Record the positive control slide staining result on mouse database DCF for each embryo in experiment. Enter the slide ID In the positive control slide box.

3. Per colony, select two heterozygotes with representative staining to continue with the analysis and imaging.

4. Record the staining result for each tissue required for wholemount annotation (See Appendix D - minimum terms for annotation) on the DCF as “Present” or “Not detected”.

5. If staining is present: Image tissues in 70% glycerol using a Leica MZ16A stereomicroscope (0.5x objective). Image the placenta if staining is present on the chorioallantoic tissue (placenta, yolk sac or umbilical cord)

   See Appendix D for standardised images of the embryo (2 x lateral views, dorsal, ventral view) and placenta.
   - Use petri-dish with transparent silicon base and insect pins to immobilise tissues as required.
   - Adjust the lighting, microscope diaphragm and exposure time to achieve a well illuminated sample with a uniform white background.
   - Images acquired using Leica LAS v4.4 software
     - In the form “Image Data” scan in the correct annotations for the image. The annotations will carry over to the next image so be careful to check the annotations are correct before acquiring the image:
       i. **Image Name:** Enter the mouse name e.g. XXXX12.3a_
       ii. **Description:** Wholemount Expression
       iii. **Notes:** Embryo or Placenta (as appropriate)

6. Annotate the pictures on the mouse database using EMAP terms.

Glycerol waste must be disposed into appropriately labelled waste containers and given to the Sanger Waste Team for disposal.

PROCEDURE E: Freezing of fixed embryos
One person should avoid freezing more than 2 litters at a time to avoid compromising the freezing quality.

1. **In a fume hood** place a cold-proof metal container into an ice bucket and surround with dry ice.

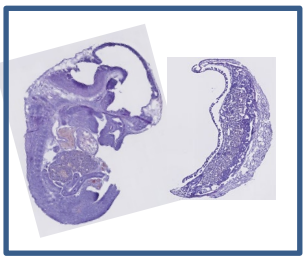
2. **Pour isopentane** into the metal container
   a. Place a thermometer (on top of an embedding mould so as to not touch the bottom of the container)
   b. Add dry ice to the isopentane until the temperature measures -60°C. This was established during pilot work (do not use a lower temperature).

3. Fill a small embedding mould with OCT. Take care to remove all the bubbles from the OCT.

5. Print small labels with sample ID to be attached to the OCT block.

4. Tip the embryo and placenta out of the bijou tube, onto a perforated moria spoon and remove as much sucrose as possible using blotting paper.
   Do not put the embryo or placentas directly onto the blotting paper; instead carefully wick away liquid with the edges of the paper.

5. Using room temperature forceps, quickly place the embryo and placenta in the mould (orientation below; embryo is placed with its left side down).



6. Place ID label on top of the OCT (do not cover the label in OCT or you will not be able to read it once frozen).

7. Place the mould directly into the isopentane until it touches the bottom.

8. Submerge the mould in isopentane for ~1 minute and 30 second until the OCT is frozen throughout the block.

9. Leave block on dry ice until isopentane evaporates. Store the OCT blocks from 1 litter, in a 50 ml falcon tube, in a -80 freezer. Place in rack "For sectioning" and record location on laminated sheet on freezer.

Wearing cold-resistant gloves, carefully remove the metal container of iso-pentane from the dry ice. The iso-pentane and dry ice can be left with the lid off, in a fume hood or downflow table to evaporate – ensure it is clearly labelled.

Alternatively, the iso-pentane can allowed to warm to -10C, then decanted to a suitable bottle for re-use. The bottle must be labelled and kept in a fume hood/ downflow table, with the lid loosened until the iso-pentane returns to room temperature.

Dry ice can be left to evaporate on a down-flow or fume hood.

PROCEDURE F – Cryosectioning of fixed frozen embryos

Cryosectioning

Per colony: Section two heterozygotes from different litters. Aim to section them on the same day. If a line cannot be completed in one session, record this on the review Lacz spreadsheet.

Section 1 WT from the first litter collected each month, to check for endogenous LacZ stain.

1. Label slides with the mouse id and slide number. Mark slides 3, 6, and 9 (Indicated below in red) with an asterisk for non-counterstaining.

2. Section at 30um. Collect all sections (3 sections per slide).
   Arrange the first 24 sections across 8 slides, and the last 12 sections across 4 slides.
   Collect a total of 12 slides (should section most of the way through the block).

	Slide 1	Slide 2	Slide 3 *	Slide 4	Slide 5	Slide 6 *	Slide 7	Slide 8
Section #	1	2	3	4	5	6	7	8
	9	10	11	12	13	14	15	16
	17	18	19	20	21	22	23	24

	Slide 9 *	Slide 10	Slide 11	Slide 12
Section #	25	26	27	28
	29	30	31	32
	33	34	35	36

3. Place slides in a slide box labelled with the date of sectioning, the mouse line and initials of the sectioner (keep slides 3,6 and 9 separate in the box). Store at -80°C.

4. Update the biobank status on the DCF for each sectioned embryo to 'unstained sections'.

5. The remaining unsectioned blocks from a litter should be stored at -80°C, in a rack labelled "For Biobanking".

6. Per colony: a maximum of 3 Hets, 3 WT unsectioned blocks were selected for biobanking (DCF consulted to avoid damaged or atypical embryos where possible).
   Blocks archived in 50ml falcons, at -80°C alphabetically by gene.

PROCEDURE G: LacZ staining of cryosections

LacZ staining

1. Remove slides and a positive control slide from -80°C freezer and allow to defrost for a minimum of 3 hours.
   a. Label positive control slide with the experiment as "frozen" and the date of stain in pencil.

2. Place slides into slide rack.

3. **In a fume hood** make up 4% PFA solution and refrigerate.

4. **In a fume hood** place slide rack into staining boxes and fix the slides in cold 4% PFA for 10 mins.

5. Remove fixative and wash slides in cold PBS (pH8), 5 mins.

6. Perform 2x washes in PBS (5mins each).

Washes need to be gentle (otherwise lose sections/ sections lift) but thorough (otherwise you get LacZ crystals).

PFA waste and washes must be disposed into the PFA waste container and given to the Sanger Waste Team for disposal.

7. **In a fume hood** make up LacZ solution (excluding x-gal) in a glass bottle (150 ml per staining rack - 25 slides).
   Add the X-gal just before use. Ensure the LacZ solution is thoroughly stirred using a magnetic stirrer. (See Appendix B).

   (For details of positive control staining, see Appendix C).

8. Incubate slide rack in LacZ staining solution at 37°C in hybridisation oven, overnight. Keep dark during staining, by using lidded black staining boxes.

Post-fixation, counterstaining and coverslipping

9. Filter the Nuclear Fast Red counterstain through Whattman paper, to remove any precipitate.

10. **In a fume hood**, remove slides from LacZ staining solution and wash with PBS.

    LacZ waste and washes must be disposed into the LacZ waste container and given to the Sanger Waste Team for disposal.

11. **In a fume hood**, fix the slides in 4% PFA and counterstain with Nuclear Fast Red (NFR) before dehydrating through an ethanol series, xylene clearing and mounting.

    Slides may be coverslipped manually **in a fume hood**, or using an automated coverslipper.

Note that slides #3, 6 and 9 in a section series should not receive counterstaining.
Steps highlighted in blue are for counterstained slides only.

Step	Reagent	Duration (mins)	
1	4% PFA - cold	10	
2	PBS pH8 rinse	5	
3	Ultra-pure sterile water x2 washes	2	Gentle rinses or will lose sections.
4	NFR stain	Rapid dip	(<2 sec)
5	Ultra-pure sterile water x 3 washes	3-5 mins	Gentle rinses: Gently lift rack from one clean bucket to another (approx 30 sec per bucket/until water runs clear.
6	70% Ethanol	2	
7	100% Ethanol x2	2	Replace with fresh ethanol after every rack.
8	Xylene x 2	1	Replace with fresh Xylene after every rack.
9	Mount		

PFA waste and washes must be disposed into the PFA waste container and given to the Sanger Waste Team for disposal.

Ethanol and Xylene waste must be disposed into the Ethanol and Xylene waste container and given to the Sanger Waste Team for disposal.

PROCEDURE H: Analysis and imaging of LacZ stained slides

Slide Imaging

1. Examine the slides under a microscope for the presence or absence of LacZ staining and section quality.

2. Per embryo, select sections for imaging that represent all the tissues on the minimum annotation list (Appendix E).

   Sections should be of publishable quality:
   a. No bubbles/crystals
   b. Aim for “whole sections” organs not folded over
   c. Small creases/tears are permissible
   d. Ideally, skin/spinal cord/cranium should not be coming away from the section

3. Once selected, print labels for slides and image using the Hamamatsu Slide Scanner.

Slide annotation

4. In the DCF, record the staining result for each tissue listed on the minimum annotation list (Appendix E) as “Present” or “Not detected”.

   If the staining pattern is typical of the endogenous staining seen in wild-type embryos, record it as “Not detected”. Record an “Ambiguous” staining result where the staining result is uncertain e.g. very faint, non-discrete staining but atypical for endogenous staining.
   For examples of endogenous LacZ staining in wild-type embryo cryosections, refer to lab WT image example catalogue.

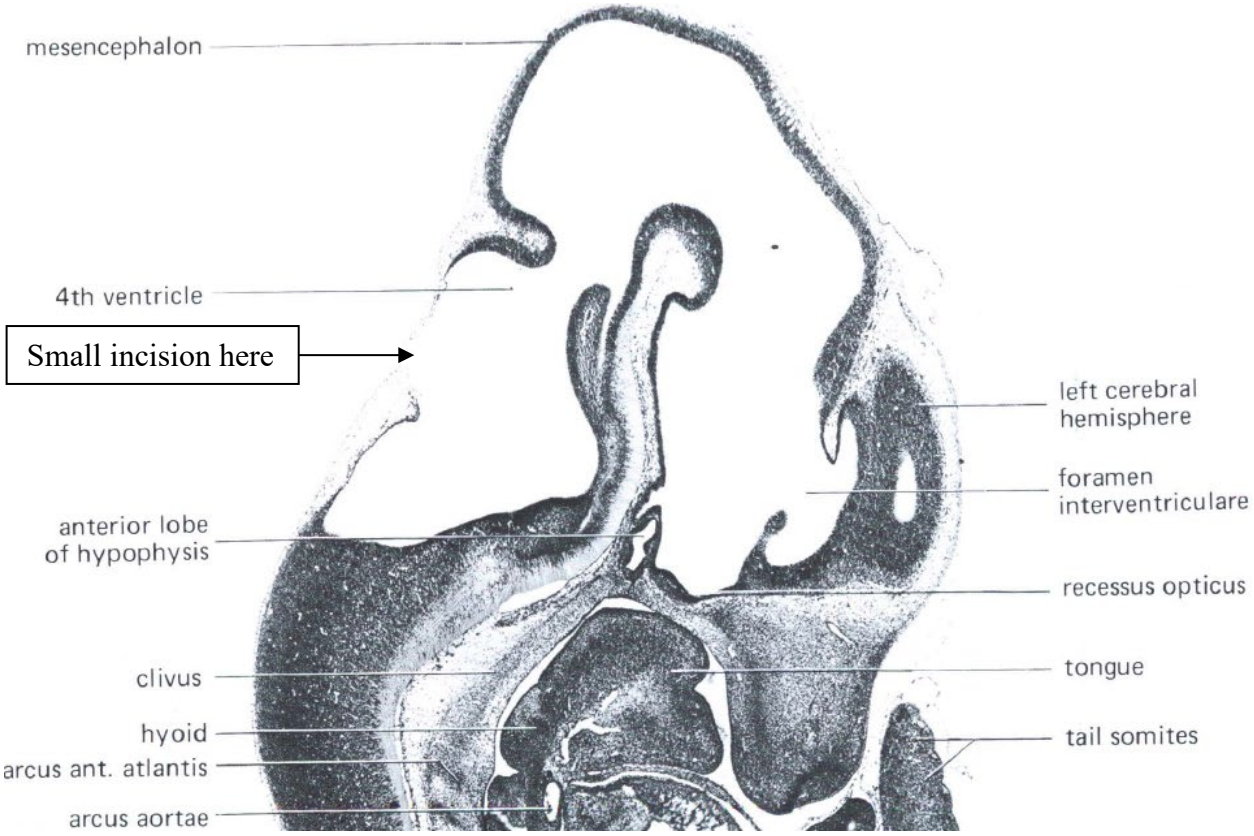
7. Record the positive control, slide staining result on mouse database DCF for each embryo in the experiment. Enter the slide ID In the positive control slide box.

8. Annotate the slide images on the database using using MA ontology OLS to show examples of LacZ staining in tissues from the minimum annotation list.

9. Once annotation is complete, slides can be archived alphabetically by Gene name.

Appendix A: ED12.5 dissection

Sagittal section through a 12 day old mouse embryo. Theiler, K. 1989. The house mouse – Atlas of Embryonic Development. Springer-Verlag.



APPENDIX B – Reagents:

Fixation and washes

PBS from 10x stock solution

For 1L 1xPBS; mix 100mL 10xPBS with 900mL water

- Adjust to pH 8.0 with sodium hydroxide

Sodium hydroxide causes damage to organs and is corrosive.
To be used only under an effective fume hood or on a functional downflow table wearing correct PPE.

4% Paraformaldehyde (solution stock 32%)

PFA is toxic, harmful, corrosive, an irritant, a skin sensitizer, possibly carcinogenic and can cause damage to organs. To be used under an effective fume hood and wearing correct PPE.

For 1L 4% PFA; mix 125mL 32% PFA stock with 875mL cold PBS pH8.0.

Glycerol for clearing and storage of wholemount samples

50% Glycerol in PBS pH7.4

For 1L 50% Glycerol; Mix 500mL Glycerol and 500mL PBS pH7.4 overnight on a stirrer.

- Note: Add the PBS first, start the stirrer and gradually add the glycerol.

70% Glycerol in PBS pH7.4

For 1L 70% Glycerol; Mix 700mL Glycerol and 300mL PBS pH7.4 overnight on a stirrer.

- Note: Add the PBS first, start the stirrer and gradually add the glycerol.

70% Glycerol in PBS pH 7·4 with 0·01% Sodium azide

For 1L 70% Glycerol; Mix 700mL Glycerol and 300mL PBS pH7.4. Add 1mL 10% sodium azide. Mix overnight on a stirrer.

- Note: Add the PBS first, start the stirrer and gradually add the glycerol.

10% Sodium Azide – stock solution

Sodium Azide is toxic and an irritant. To be used only under an effective fume hood wearing correct PPE.

For 100mL 10% sodium azide stock solution; 10g sodium azide in 100ml RO water. Mix thoroughly on a stirrer.

Stock reagents for LacZ staining

4% X-Gal stock solution in DMF (40 mg/ml) – stock solution

Dimethylformamide (DMF) is harmful, flammable, causes damage to organs and an irritant. To be used under an effective fume hood and wearing correct PPE. Use glass cylinders as can melt plastic.

Dissolve 1g X-gal in 25mL DMF.

- Store in a foil covered bottle in the dark at -20°C.

10% Deoxycholic acid in water – stock solution

Deoxycholic acid is difficult to dissolve and requires stirring for several hours before aliquoting and storage at -20°C.

1. For 100mL 10% deoxycholic acid; Measure 10g deoxycholic acid in 100ml RO water. Mix thoroughly on a stirrer.
2. Aliquot into 1-2 ml eppendorf tubes. Store at -20°C.

- Note: use a 250ml beaker and get some of the water stirring before adding the deoxycholic acid.

The following LacZ staining stock solutions should be changed either monthly, or when the potassium ferrocyanide solution becomes yellowish instead of colourless – whichever occurs soonest:
$MgCl_2.6H_2O$, IGEPAL, Potassium Ferrocyanide, Potassium Ferrocyanide.

200mM $MgCl_2.6H_2O$ in water – stock solution

FW: 203.31g/mol for 100mL of sol. stock 200mM: 4.07g - store at 4C
for 50mL of sol. stock 200mM: 2.04g - store at 4C

For 100mL 200mM $MgCl_2.6H_2O$ stock solution; in a 100mL bottle put 4.07g $MgCl_2.6H_2O$ and make up the volume to 100mL with water. Mix thoroughly on a stirrer.

2% IGEPAL (CA-630) in water – stock solution

For 100mL 2% IGEPAL stock solution; in a 100mL bottle put 2mL IGEPAL and make up the volume to 100mL with water. Mix thoroughly on a stirrer.

100mM Potassium ferrocyanide II ($K_4Fe(CN)_6 \cdot 3H_20$) in PBS pH8 – stock solution

FW: 422.41g/mol 100mM stock: 42.24g/L
Light sensitive - store at 4°C.

For 500mL 100mM Potassium Ferrocyanide stock solution; in an amber 500mL bottle put 21.12g Potassium Ferrocyanide and make up the volume to 500mL with PBS pH8.0. Mix thoroughly on a stirrer.

100mM Potassium ferricyanide III ($K_3Fe(CN)_6$) in PBS pH8 – stock solution

FW: 329.24 g/mol 100mM stock: 32.92g/L
Light sensitive - store at 4°C.

For 500mL 100mM Potassium Ferricyanide stock solution; in an amber 500mL bottle put 16.46g Potassium Ferricyanide and make up the volume to 500mL with PBS pH8.0. Mix thoroughly on a stirrer.

LacZ staining solution - Wholemount:

6mL per embryo (in 7ml bijou) + 10ml for positive control

		Number of embryos:									
		1	2	3	4	5	6	7	8	9	10
2mM MgCl2.H2O	uL	160µl	220µl	280µl	340µl	400µl	460µl	520µl	580µl	640µl	700µl
0.02% IGEPAL	uL	160µl	220µl	280µl	340µl	400µl	460µl	520µl	580µl	640µl	700µl
5mM potassium ferrocyanide	mL	800µl	1.1	1.4	1.7	2.0	2.3	2.6	2.9	3.2	3.5
5mM potassium ferricyanide	mL	800µl	1.1	1.4	1.7	2.0	2.3	2.6	2.9	3.2	3.5
Cold PBS pH 8.0	mL	14	19	24	29	34	39	44	50	55	60
0.01% deoxycholic acid	ul	16µl	22µl	28µl	34µl	40µl	46µl	52µl	58µl	64µl	70µl
0.1% (1mg/ml) X-Gal in DMF	mL	400µl	550µl	700µl	850µl	1000µl	1.2	1.3	1.5	1.6	1.8
Total volume	mL	16	22	28	34	40	46	52	58	64	70

LacZ staining solution - Cryosections:

150 mL per staining box

		Volume of LacZ staining stock solution					
		1 box	**2 Boxes**	**3 Boxes**	**4 Boxes**	**5 Boxes**	**6 Boxes**
2mM MgCl2.H2O	mL	1.5	3	4.5	6	7.5	9
0.02% IGEPAL	mL	1.5	3	4.5	6	7.5	9
5mM potassium ferrocyanide	mL	7.5	15	22.5	30	37.5	45
5mM potassium ferricyanide	mL	7.5	15	22.5	30	37.5	45
Cold PBS pH 8.0	mL	128	256	384	512	641	769
0.01% deoxycholic acid	mL	150ul	300ul	450ul	600ul	750ul	900ul
0.1% (1mg/ml) X-Gal in DMF	mL	3.8	7.5	11.3	15	18.8	22.5
Total Volume	mL	150	300	450	600	750	900

APPENDIX C: Positive control slides

10. Label a positive control slide in pencil with accompanying LacZ staining experiment (either wholemount "WM", or fixed frozen sections "frozen) and the staining date.

11. For Wholemount experiments:
    In a fume hood place slide in a 50ml falcon tube with remaining 10ml LacZ staining solution, ensure tissue section is covered. Incubate upright, overnight at 37°C in an oven (keep dark).

    For fixed frozen sections: **In a fume hood** place slide in rack containing other slides for staining. Place rack in staining container and cover with LacZ staining solution, incubate overnight at 37°C in an oven (keep dark).

12. **In a fume hood** after staining remove slide from LacZ stain. Wash in RO water before examining under microscope for staining result.

    LacZ stain waste, paraformaldehyde waste and glycerol waste must be disposed into appropriately labelled waste containers and given to the Sanger Waste Team for disposal.

13. For long term storage, **in a fume hood** fix slide in 4% PFA and counterstain with Nuclear Fast Red before dehydrating through an ethanol series, xylene clearing and mounting.

Step	Reagent	Duration (mins)
1	4% PFA	10
2	RO water washes x3	3-5 mins
3	NFR counterstain	Rapid dip
4	RO water washes x3	3-5 mins
5	70% Ethanol	2
6	100% Ethanol x2	2
7	Xylene x2	1
8	Mount	

14. Once dry, print label for slide and scan using the Hamamatsu Slide Scanner.

15. In comment box on slide image on mouse database, record staining date and staining experiment "Wholemount".

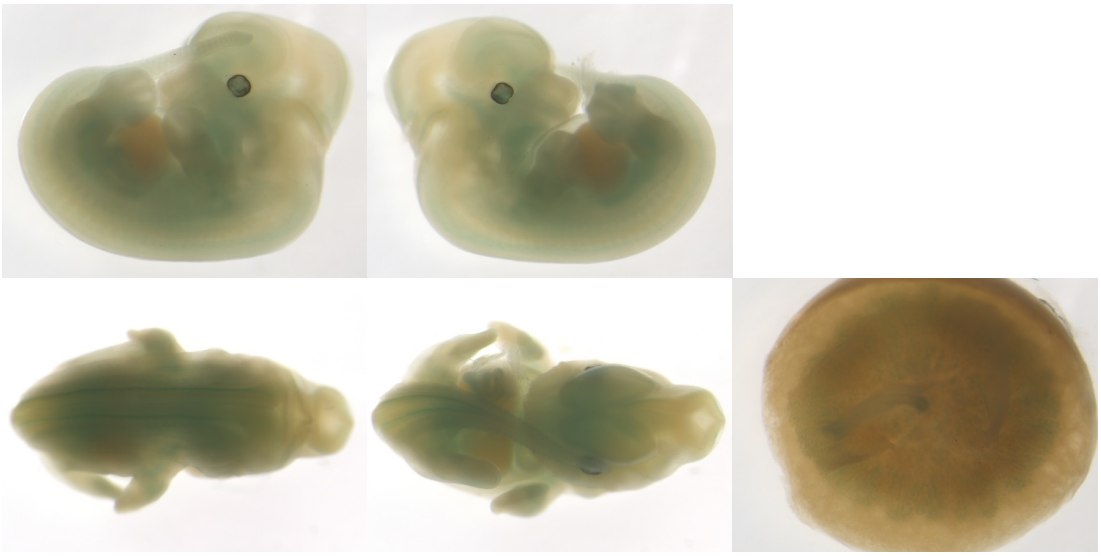
16. Record positive control slide staining result on mouse database DCF for each embryo in experiment. Enter the slide ID in the positive control slide box.

17. Positive control slides are archived chronologically by staining date.

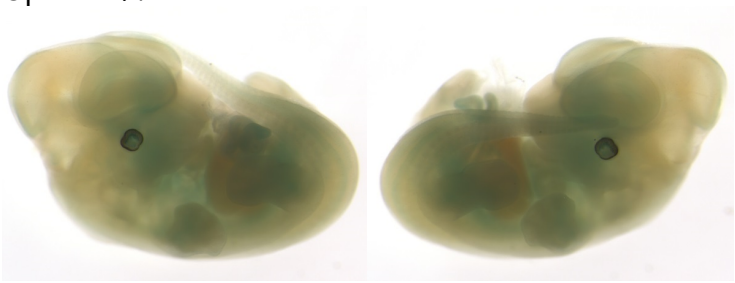
Appendix D: Minimum annotation and Imaging for wholemount LacZ stained embryos:

Minimum annotation terms:	Optional terms:
Embryo	Eye
Placenta	Heart
Head	
Forelimb	
Hindlimb	
Tail	
Ear (Pinna)	

ED12.5 Minimum Images if staining present:



Optional ¾ views:



Appendix E: Minimum annotation list for cryosectioned, LacZ stained embryos

Annotation Feature	EMAP ID:TS20	Frozen
Embryo		Yes
Placenta	EMAP:31997	Yes
Head	EMAP:31902	optional
Brain	EMAP:4126	Yes
Forebrain	EMAP:4127	Yes
Hindbrain	EMAP:4187	Yes
Midbrain	EMAP:4219	Yes
Spinal cord	EMAP:4251	Yes
Dorsal root ganglion	EMAP:4276	Yes
Ear	EMAP:4286	Yes – inner ear
Eye	EMAP:4327	Yes
Oral cavity	EMAP:3985	Yes
Mandibular process (Lower jaw)	EMAP:4552	Yes
Maxillary process (Upper jaw)	EMAP:4557	Yes
Lung	EMAP:4604	Yes
Heart	EMAP:4396	Yes
Stomach	EMAP:4492	Yes
Liver	EMAP:4577	Yes
Skin	EMAP:4147	Yes
Forelimb	EMAP:4012	optional
Hindlimb	EMAP:4058	optional
Tail	EMAP:4651	optional (if genotyped by YS)
Somite	EMAP:4655	Yes (if genotyped by YS)