SANGER INSTITUTE
STANDARD OPERATING PROCEDURE

SUBJECT: Neonate Phenotypic Assessment P0-P3

INTRODUCTION:

The purpose of this procedure is to assess new-born pups for parameters that are essential to neonate life. These include: gross dysmorphology, breathing assessment, skin coloration and cyanosis, weight, crown rump length, tail length, righting reflex, ability to move, blood glucose level, and milk spot morphology. Toe clips will be performed for identification and to provide a genotyping sample.

Neonates will be culled by overdose of Pentobarbital Sodium and processed for histopathology or uCT analysis or returned to the dam for up to 7 days. Pups will not be kept alive past P9.

Staff requirements: **Two staff members are required to perform this task.**

Normal Workflow: Both staff members will be competent in all tasks and rotate Person 1 and Person 2 with each mouse

Backup Workflow: Person 1 must be fully competent in all regulated procedures
Person 2 must have a PIL and can handle mice and use the database
Staff members will not rotate roles

This is a regulated procedure.

QUALITY CONTROL (QC) DURING PROCEDURE:

Problem / Issue	Comment on DCF / action to be taken
A welfare issue makes it impossible to perform the procedure	Do not perform procedure. If necessary, cull distressed mice (see section E). If possible, take genotyping samples, and fix neonate in formalin (see section D) Record the welfare issue on DCF.
Procedure affected due to fire alarms	Stop procedure, and return mice to the home cage on a holding rack. Return and complete procedure when/if it safe to do so.

HEALTH & SAFETY:

This procedure is covered by the following risk assessments: **WTSI_2877 & WTSI_2878**

- Entry procedure to the Animal house should be followed.
- Appropriate PPE is to be worn at all times when handling animals. This includes:
    - Overshoes
    - Gown
    - Gloves

- Access to a functional down-flow table is required
- New Workers are to be supervised until deemed competent to perform this assay
- When sources for 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.

- Eye wash stations check your local area for locations. Fire extinguishers a check your local area for locations; standard spill kits check your local area for locations and a solvent/formaldehyde spill kit check your local area for locations.
- Lone worker alarms should be used when working alone.
- This procedure can only be performed during **Animal facility core hours** (7:30am-7:30pm).
- All electrical equipment is to be inspected for damage before use.

All staff performing this procedure are responsible for ensuring that this Standard Operating Procedure (SOP) and accompanying Risk Assessment have been read, understood and where applicable is 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:

1. Appropriate PPE (see above)
2. Computer with database access and cage card printer
3. Data recording form
4. Heat mat (to place cage onto)
5. Heat mat (to phenotype on)
6. Cork board x2 (to phenotype on)
7. Ruler + Measuring Grid
8. 5cm petri dishes
9. Timer x2
10. Weighing scales and pot (seeded with bedding from home cage)
11. Dissection tools
12. Spring scissors (Fine Science Tools; Vannas-Tübingen Spring Scissors; 15004-08
13. Forceps (Fine Science Tools; Dumont #3 Forceps)
14. Genotyping plates, seals, and cold 96 well racks
15. Glucose meter x2 (ACCU-CHEK Aviva Kit; 3171261)
16. Glucose meter strips (ACCU-CHEK Aviva Test Strips; 3171253)
17. Control solutions (ACCU-CHEK Aviva Control Solution; 3171246)
18. Ethanol wipes
19. Cotton wool buds
20. Microscope and camera
21. Dysmorphology camera
22. 200mg/ml Pentobarbital Sodium (Euthatal®solution- Merial 08327/4112)
    Pentobarbital sodium is toxic. Correct PPE must be worn.
23. 0.5ml syringes (Terumo Myjector U100 Insulin Syringe 29G x ½" 0.5ml BS=05M2913)
24. Body bag
25. Small scalpel blade
26. Sharps bin
27. Paintbrush and warm PBS
28. 60ml Formalin pots (Cellpath BAF-6000-08A)
    Toxic, corrosive, carcinogenic, skin and respiratory sensitizer, causes damage to organs and an irritant substance that contains formaldehyde. Storage in fixatives safety cabinet. Must be handled on a down-flow table and wearing correct PPE.

PROCEDURE:

Before starting this procedure, check the documentation to identify expectant and existing litters that require phenotyping.

Note: Keep pup handling at a minimum. If to be performed on multiple litters, gloves must be sprayed with Hydrex Pink hand spray and the working area must be cleaned with Hydrex

Hard surface spray between litters, so as to reduce the transfer of smell and reduce the chance of rejection.

1. Set Up:

1.1. **Set up work area:**

1.1.1. Heat mat for home cage
1.1.2. Heat mat on cork board for working area
1.1.3. Measuring grids
1.1.4. Petri dishes and timer (for righting response)
1.1.5. Weighing scales and pot seeded with home cage bedding
1.1.6. Dissection tools – forceps and scissors
1.1.7. Genotyping plates
1.1.8. Data entry cards

1.2. **Calibrate and QC glucose meters:**

Calibration should be performed at the start of every session and again if a new tube of test strips is started.

1.2.1. Insert a glucose strip into the meter
1.2.2. Wait until the 'ready for sample' symbol flashes
1.2.3. Squeeze a drop from control solution L1 onto the strip (best to do horizontally)
1.2.4. Record the reading on form and remove the strip
1.2.5. Press the right arrow key to select 'L1' and then press the on/off/set button. There should be an 'OK' message
1.2.6. Repeat with second L2 solution to set L2 value

1.3. **Transfer home cages from:**

1.3.1. Locate cages in the animal room and sign cages out of room
1.3.2. Transfer cages to experimental room
1.3.3. Keep on cage holding rack until ready to start phenotyping

2. Home Cage Assessment:

2.1. **Transfer first home cage** to heat mat in working area
Open cage and assess the **state of the nest:**

Full Dome	*High walls with a central hollow enclosed*
Incomplete Dome	*Walls reach widest point of a sphere shape*
Cup shaped	*Walls for a shallow cup/bowl shape*
Flat	*Clear nest area, but no walls*
No Nest	*No Nest*

2.2. **Count the pups in the cage:** Compare to expected number of pups. If litter size does not match what was expected for the cage proceed to step 2.2.1. If there are no newly discovered, missing, or found dead pups, proceed to phenotyping (step 3).

2.2.1. **If there are more pups than expected, add new pup to the database:**

2.2.1.1. Search for litter from home page
2.2.1.2. 'Edit Litter' and update '#Born' value and click 'Save'
2.2.1.3. Add pup to cohort
2.2.1.4. If litter is to be culled at P0, add '**Neonate Daily Checks P0-P9**' procedure to new pup
2.2.1.5. If litter is to be taken past P0, create a '**Neonatal Screen Daily Checks**' DCF for the new pup

2.2.2. **If there are fewer pups than expected <u>search the cage thoroughly.</u>** Missing pups may be hidden in the nest or bedding. If pup can't be found, record as missing on the database:

2.2.2.1. If litter has previously been identified, use toeclips of remaining pups to identify which pup is missing. If litter has not yet been toeclipped, start recording missing pups in ascending alphabetical order beginning with .1a. (See appendix for toe clipping)

2.2.2.2. Cull pup on the database, recording 'Fate' as 'Missing'

2.2.3. **If there are pups found dead:**

2.2.3.1. Remove dead pup from the cage immediately. If pup is not whole, remove as much as possible

2.2.3.2. Identify the pup using its toeclip, or give it a toeclip identification starting at .1a. Use the toeclip sample for genotyping. (See appendix for toe clipping)

2.2.3.3. Cull pup on the database, recording 'Fate' as 'Found Dead'

2.2.3.4. Perform as much of a Neonate Assessment as possible on the dead pup Record where possible: Sex, Lengths, Weight, Dysmorphology Comments

2.2.3.5. If pup is not intact and this impairs a measurement then do not take the impaired measurement.
For example: if the head is missing, do not measure Crown to Rump length or Weight.

3. Visual Assessment of Neonate Welfare, Gross Dysmorphology and Movement

If during visual assessment of the pups, there are any moderate or substantial signs of stress (see **Section 13**), the distressed pups should prioritised and culled as soon as possible.
Neonates in **Moderate** distress should be phenotyped and culled.
Neonates in **Substantial** distress should be culled and then phenotyped post-mortem.

3.1. **Person 1:** Take out first pup; perform a **visual welfare assessment** and record:

3.1.1. **Ability to breathe** (normal or gasping)

3.1.2. **Skin colouration** (signs of cyanosis)
If pup is pale or blue in colour, use a paintbrush with warm PBS to try and stimulate the pup to breath

3.2. **Person 1:** Perform the **Righting Response**

3.2.1. Place pup in 5cm petri dish

3.2.2. Cover dish with laminated grid, to form a tight seal. Ensure the pup makes contact with the base of the dish with all 4 paws, when inverted upside down

3.2.3. Place inverted dish on a flat surface, remove top and start the timer

3.2.4. Observe pup for **20 seconds** and score their ability to right themselves:

0 = No attempt at righting
1 = Struggles - pup rocks back and forth or moves limbs in an attempt to right itself
2 = Attempts - 1 paw touches the floor
3 = Almost - 2 or 3 paws touch the floor
4 = Fully - all 4 paws make full contact with the floor
If Score = 4, also record the time taken for pup to fully right itself

3.3. **Person 1:** Perform a **visual dysmorphology assessment** and record:

3.3.1. General **gross dysmorphology** (see DCF for full list)

3.3.2. Capacity for **movement**

3.3.3. Presence and size of **milk spot**

3.3.4. **Sex** of mouse

3.3.5. Take photos if necessary

4. Measurements & Weights:

4.1. **Person 1**

4.1.1. Using the 2mm grid, record the **crown-rump length** and **tail length** of the pup

4.1.2. Place pup on scales and **record weight**

5. Blood glucose Concentration:

5.1. **Person 1**

5.1.1. Scruff mouse

5.1.2. Using the small, spare scissors, take a **small tail tip**

5.1.3. **Collect tail sample in a genotyping plate**

5.2. **Person 2**

5.2.1. Insert a **glucose strip into the meter**

5.2.2. Wait until the 'ready for sample' symbol flashes

5.2.3. Position the strip to collect a **drop of blood from the tail tip**

5.2.4. Record the result

Dispose of the glucose strip in the sharps bin

5.2.5. If there is **not enough blood** for a reading (Error E4), take a second tail tip

Two attempts is the maximum allowed, after this, record as unsuccessful

If pup bleeds, place onto a paper towel to absorb the excess blood.

If pup continues to bleed after the sample has been taken, place onto a paper towel. Bleeding should stop in < 1 minute. If blood does not start to clot, or pup becomes inactive and unresponsive, pup should be culled.

6. Toe Clip for Identification (See Appendix ..):

6.1. **Person 1**

6.1.1. Scruff mouse

6.1.2. Use Appendix to identify which toe clip needs to be taken

6.1.3. Use **small curved scissors to remove the toe**

6.1.4. **Collect toe sample in a genotyping plate**

6.1.5. **If pup bleeds, place onto a paper towel to absorb the excess blood**.

If pup continues to bleed after the sample has been taken, place onto a paper towel. Bleeding should stop in < 1 minute. If blood does not start to clot, or pup becomes inactive and unresponsive, pup should be culled.

7. Pup Retrieval:

7.1. **Person 1**

7.1.1. Return pup to home cage, and place at the opposite side of the cage to the dam/nest

7.1.2. Start timer and observe

7.1.3. **Record how long it takes the dam to retrieve the pup back into the nest**

7.1.4. Record any abnormal behaviour of the dam

7.1.5. If the dam has not retrieved the pup in **2 minutes**, open cage and place pup into nest

7.2. **Person 2**

7.2.1. Take the next pup from the cage

7.2.2. Repeat steps 3-7 for each pup, with staff members switching roles

7.3. Once all pups in the cage have been phenotyped, return cage to holding rack

Clean tools, cork board, weight pot and surfaces with antibacterial solution or alcohol wipes

8. **If pups are being returned to the dam:**
   8.1. Follow Data Capture and Genotyping steps below (Steps 10 & 11)
   8.2. Print new cage card
   8.3. Check procedure has been added correctly on cage card
   8.4. Return cage to Yellow 5

9. **If pups are being terminally collected:**
   9.1. Leave on holding rack until ready to cull
   9.2. Follow Data Capture and Genotyping steps below (Steps 10 & 11)

10. **Data Capture:**
    Starting the DCF adds a regulated procedure to the mice. The DCF must be started on the day of phenotyping. The DCF must be filled in for all mice (including any found dead). Separate DCFs must be created for living and dead pups. **Ensure pup's fate is correct on the database before creating the DCF.**

    10.1. **Neonatal Screen P0-P3 DCF**
    10.1.1. Using the data card notes, fill in the DCF for each mouse

    10.2. **Neonatal Screen Daily Checks DCF:** Pups older than P0 and pups being returned to dam after phenotyping should have a Daily Checks DCF
    10.2.1. Update life status for appropriate timepoint for each pup

    10.3. **Add Sex of Pups to Database:** If pups have been sexed previously, edit any which are incorrect and make a note of the change on the Daily Checks DCF

11. **Genotyping Plates:**

    11.1. **Create and register genotyping lists for toe and tail samples:**

    11.2. **Toe genotyping samples:**
    11.2.1. Spin samples in the centrifuge
    11.2.2. Submit both paperwork and genotyping plate to genotyping team
    11.2.3. Genotyping samples should be dropped off in freezer

    11.3. **Tail genotyping samples:**
    11.3.1. Spin samples in the centrifuge
    11.3.2. Store plate in 'Neonate Tail Plates' box in freezer

12. **Dysmorphology images:**

    12.1. **Live Pups:**
    12.1.1. Take images using the dysmorphology camera
    12.1.2. If possible, have the mouse ID visible in the photo
    12.1.3. Take images off the camera by connecting the camera to the computer and upload onto database via the manual upload function

    12.2. **Dead Pups**
    12.2.1. Use the stereomicroscope in to take images
    12.2.2. Ensure that the image name is the correct mouse name in the format MXXX1.1a_
    12.2.3. In the description box enter 'Neonate Dysmorphology'
    12.2.4. In the comments box use barcode scan sheet to enter pup age and orientation tags
    12.2.5. Check that the images have been uploaded to the database with the correct tags

Appendix A: Neonate Terminal Collection for Drop Fixation P0-P1

INTRODUCTION:

The purpose of this procedure is to cull, collect and wholemount drop fix new-born pups. Neonates are to be culled by overdose of Pentobarbital and drop fixed in 10% Formalin. Samples will then be bio-banked in preparation for further analysis, e.g. uCT or histopathology.

Staff requirements: One member of staff is required to perform this task.

PROCEDURE:

Before starting this procedure, in the majority of cases, phenotyping will have occurred as stated.

1. Set Up

i. Turn on down flow table & gather tools required
ii. Use Traka card to access the drug cabinet
iii. Place home cage on a heat mat
iv. Prepare another heat mat with a clean, empty cage on top

2. Print Labels and prepare 60ml Formalin pots

3. Pentobarbital preparation

3.1.1.1. One needle can be used to dose a maximum of 3 pups with **0.03ml per pup** (take up 0.1ml for 3 pups)
3.1.1.2. Prepare by inserting syringe needle tip into the Pentobarbital bottle, through the lid
3.1.1.3. Invert the bottle and slowly withdraw the plunger to take up the desired volume of pentobarbital
3.1.1.4. Remove any air bubbles by tapping the side of the syringe and ejecting the air out from the needle tip
3.1.1.5. Withdraw needle tip from bottle and set syringe aside. **DO NOT recap the syringe**
3.1.1.6. **Record volume of Pentobarbital used** in the Pentobarbital log book

4. Culling of the Dam

i. On a down-flow table, sacrifice the female by cervical dislocation
ii. Check that the female is unresponsive by pinching the paws or tail
iii. Damp the fur with 70% Ethanol and open up the body cavity to confirm death by removal of the heart
iv. Dissect down to investigate the uterus for any unborn pups. Any pups found should be phenotyped
v. Dispose of female in a sealed body bag and store bag in the cadaver freezer

5. Culling of neonates by IP injection
Before Proceeding: The dam must be culled before pups are culled and collected, except where a pup needs to be culled sick.

5.1.1.1. Remove pup from home cage and scruff
5.1.1.2. **Tilt pup backwards and insert the needle into the right lower quadrant of the abdomen at an approximately 45 degree angle, taking care to avoid organs**
5.1.1.3. Inject required volume and wait for 5 seconds
5.1.1.4. Withdraw the needle carefully, turning as retracting to prevent leakage
5.1.1.5. Place pup in separate cage prepared earlier during set up. DO NOT return pup to littermates in home cage

5.1.1.6. **Pup should lose consciousness and stop moving in under 1 minute**
If pup is still mobile after 3 minutes, inject a second dose of Pentobarbital

5.1.1.7. **Confirm death by:**

- Complete lack of movement and responsiveness
- Drop in temperature
- Loss of skin colouration
- On set of rigor mortis

6. Cage Check

After all pups have been culled, a final check of the cage is essential to ensure there are no hidden pups, alive or dead.

6.1.1. Perform a manual check of the nest and bedding

6.1.2. Visually check the sawdust through the cage walls, including underneath the cage

6.1.3. **Only if there has been a discrepancy in the number of pups at any point:** Perform a manual check of the sawdust

7. Genotyping Samples

If genotyping samples need to be taken, take toe/tail/ear as directed in SOP0188

8. Drop fixation in 10% Formalin

i. **On a down-flow table**, open a 60ml pot of 10% Formalin
ii. Double check label on the pot
iii. Double check the neonate toe clip identification
iv. Using a small blade, make two incisions, one on each flank of the pup, above the leg, cutting through the skin only. This is important to allow penetration of fixative.
v. Drop fix neonate in formalin pot. Transfer samples to the Lab for storage

The residual animal carcass must be disposed of in a cadaver waste bag, sealed, and put in the dedicated freezer. Clean equipment and surfaces. All dissection instruments should be scrubbed clean in the sink with antibacterial solution. Transfer all other waste to a yellow offensive waste bag or clearly labelled waste container.

9. Cull Dam and Litter on the Database

10. Data Capture

10.1.1. Ensure that the **Neonatal Screen P0-P3 DCF** has been correctly filled out

10.1.2. **If litter is >P0,** Ensure that the **Neonatal Screen Daily checks DCF** has been correctly filled out

11. Welfare Assessment Criteria

If any dam exhibits 3 or more mild signs, or a moderate sign of pain/distress, she should be culled immediately.

Pups that are immobile, unresponsive, or cold to touch should be culled immediately.

If in doubt, please check with a NACWO.

11.1. For Neonates:

Mild (observe progress)	Moderate (Phenotype and Cull)	Substantial (Cull Immediately)
Growth retarded Reduced weight gain Consistent lack of milk spot (<P4)	Sustained weight loss Sustained signs of dehydration	Weight loss > 20%
No gross dysmorphologies	Kyphosis (abnormal body curvature) Abnormal craniofacial morphology	Abnormal brain morphology Omphalocele (Herniation of intestines)
Normal skin colouration Normal respiration Partial Piloerection (>P4)	Cyanosis, with response to stimulus Intermittent abnormal breathing Marked piloerection (>P4)	Cyanosis, without response to stimulus Laboured respiration Marked piloerection (>P4) with signs of dehydration

11.2. For Dams: See table below

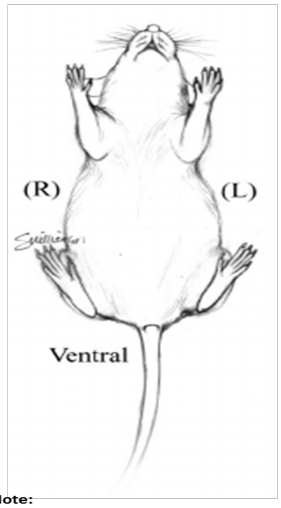
Pain and Distress in Laboratory Rodents: Guidelines for determining humane endpoints in protocols

Mild	Moderate	Substantial
• Reduced weight gain • Food and water consumption 40-75% of normal for 72 hours	• Weight loss of up to 20% • Food and water consumption less than 40% of normal for 72 hours	• Weight loss greater than 25% • Food and water consumption less than 40% for 7 days, or anorexia (total inappetence) for 72 hours
• Partial Piloerection	• Staring coat – marked piloerection	• Staring coat – marked piloerection with other signs of dehydration such as skin tenting
• Subdued but responsive, animal shows normal provoked patterns of behaviour • Interacts with Peers • Hunched transiently especially after dosing • Transient vocalization	• Subdued animal shows subdued behaviour patterns even when provoked. • Little peer interaction • Hunched intermittently • Intermittent – vocalization when provoked	• Unresponsive to extraneous activity and provocation • Hunched persistently • 'Distressed' – vocalization unprovoked
• Oculo-nasal discharge transient • (typically signs of chromorhino-dacryorrhoea in rodents) • Normal respiration	• Oculo-nasal discharge persistent • Intermittent abnormal breathing pattern	• Oculo-nasel discharge-persistent and copious • Laboured respiration
• Transient tremors • No convulsions • No prostration • No self mutilation	• Intermittent tremors • Intermittent convulsions • Transient prostration (less than 1 hour) • No self mutilation	• Persistent tremors • Persistent convulsions • Prolonged prostration (more than 1 hour) • Self mutilation

Appendix B: Toe clipping

1.1. Using small, curved forceps, place the blades either side of the toe.
1.2. Remove the toe with 1 clean cut.
1.3. Hold the pup for a few seconds to evaluate the bleeding. Place the pup on a paper towel to absorb excess blood and observe. If necessary, use a cotton wool bud.
1.4. Bleeding should stop within 1 minute. If there is excess bleeding, or the pup becomes immobile and unresponsive, the pup should be culled on welfare grounds.
1.5. Return pup to home cage and repeat with next littermate.
1.6. Once all pups have been toes clipped, place a Post Procedure check card on front of the cage label. Refer to Appendix 2.
1.7. Return cage to rack.

Toe Clipping for Identification

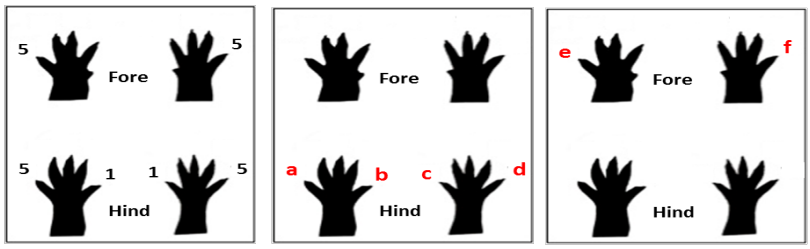


Note:
- * indicated an extra toe clip
- 'No clip' is not used, as this option will be saved for pups with digit dsymorphologies

a) RH 5
b) RH1

c) LH 1
d) LH 5

e) RF 5
f) LF 5



g) RH 5 + LH1
h) RH 5 + LH5

i) RH 5 + RF5
j) RH 5 + LR5

k) RH1 + LH1
l) RH1 + LH5