Title	Mouse Breeding Principles		
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Table of Contents

Working definition of mouse lines and strains ____ 2
General Points ____ 2
Basic Breeding ____ 2
Quality Assurance ____ 3
Problem breeding ____ 6
Monitoring ____ 6
Cull criteria ____ 6
Record keeping ____ 7
Sentinels ____ 7
Cards ____ 7
Housing of breeding stock ____ 8
Timed Mating ____ 8
Test Mating ____ 9
Cages ____ 10
Crossed lines/Experimental lines ____ 10
Nomenclature ____ 10
Breeding specific genotypes ____ 10
Backcrossing Lines ____ 11
Backcross Protocol ____ 11
Genotype Probabilities ____ 15
Pedigree Chart ____ 16

Working definition of mouse lines and strains

- Lines- Mice are bred to maintain a specified gene/s in a homozygous or heterozygous state. The line may be on a defined inbred background or mixed background. The genetic modification may result from a spontaneous mutation or human intervention.
- Strains- Generally used to denote mice resulting from a defined inbreeding or outbreeding scheme. Inbred strains are often used to provide a defined background for genetically modified lines.

General Points

- A phenotype report is required for each line and must have been approved by the AEC.
- A folder should be present in every room. This should be kept up to date.
- The folder contains:
    - A phenotype report for each line
    - Special procedures that are required for a line
    - Breeding plans for each line.
    - Production records for each line

Basic Breeding

Pairing and breeding schemes

- Mate animals at maturity eg. 7-8 weeks of age. Some lines need to be mated early because of a limited life-span due to phenotype eg. NOD/Lt mice develop diabetes and should be mated at 6-7 weeks. The optimal age for mating for most lines is 7-12 weeks. It is not recommended to pair breeders at weaning as this may delay the generation of the first litter.
- Each new pair must be given a sequential pair number. Most pairs should be permanently mated as this is the most productive breeding scheme.
- Inbred strains are paired brother to sister. If you are managing a pedigree, the pair number must be recorded on the pedigree chart. All mice used in breeding pairs must remain within 3 generations of common parents. This system can also be used for GM lines involved in simple breeding schemes to enable easy tracking of parentage. *See attachment 5 for pedigree chart.* It should be remembered that brother/sister mating can result in breeding depression so breeding performance should be closely monitored.
- If expansion is needed for inbred strains, expansion pairs can be established without the need to brother sister mate. These should no be more than 3 generations from the pedigree, and should not be used for breeding in the pedigree line.
- Outbred mice are bred using a grid pattern to ensure genetic diversity (See *Attachment 2*). Twenty breeding pairs is the minimum required to avoid inbreeding in an outbred colony.
- Random bred lines do not follow any breeding scheme. For GM lines random breeding is often used and future breeders are selected on genotype rather than parentage.
- Mice that are of a mixed or unknown wild type background should not be brother-sister mated. They are usually random mated.
- If a large numbers of mice are required in the short term, use harem system i.e. 2-4 females per male. Care must be taken to ensure the females are separated when pregnant to avoid overcrowding. *Two litters in one box is not permitted and usually results in increased pre-weaning mortalities.*

Maintaining Lines

- A minimum of 2 boxes of breeding pairs should be kept per line. In addition, 2 boxes of females and 2 boxes of males should be kept as reserve breeders.
- The 4th or 5th litters should be used to source the future breeders. Animals selected for breeding should be of the correct genotype and be in good health.

- Often lines need to be maintained as heterozygote animals. This is usually due to the homozygous genotype being lethal, or producing poor reproductive performance or impacting on the health of animals in some way. Additional boxes of replacement females and males are needed to secure the line in this case eg. 3-4 cages of females and 3-4 cages of males.
- The first litter should always be genotyped to ensure that the genotype is appropriate.
- Staggered breeding – within any one line the mating should be scattered over time unless batches of experimental mice are required.
- If the genotyping protocol can't distinguish between heterozygous and homozygous animals, the lines should be test mated every 2 years.
- Pedigree lines should be maintained for each inbred strain, so that all the animals for a line can be traced back to common parents.

Breeding life

- 6 litters per pair (or 8 months of age) unless performance is below average for the line.
- Once a female on 5th litter is visibly pregnant the male should be removed to ensure there is no post parturient mating.

Weaning

- The number of pups in a litter should be counted by 3 days. The litter must not be touched when newborn.
- At day 18 or 20 days mice should be weaned i.e. removed from the parents, separated into males and females, and food is placed in the bottom of the box. A sticker is applied to the cage card indicating the parentage of the mice. Cards need to be written before marking.
- Marking occurs as near to weaning as possible. If more than 21 days of age, general anaesthesia must be used. Marking includes identification (ear clip or implant) +/- tissue collection for genotyping.
- Before the marking day cards are written, and animal numbers are allocated. At marking the ears are clipped with the appropriate ID number ID numbers simply follow on from the number last used for that particular line.
- Mice should be sexed at least twice, once at weaning and once at marking.

Quality Assurance

Quality assurance (QA) needs to be implemented to ensure that there is no danger of lines being lost and to maintain genetic integrity.

Replace inbred strains from ARC- Every 5-10 generations, inbred lines should be replaced with new animals from ARC to minimise genetic drift in our inbred background strains.

Backcrossing lines to prevent genetic drift of background

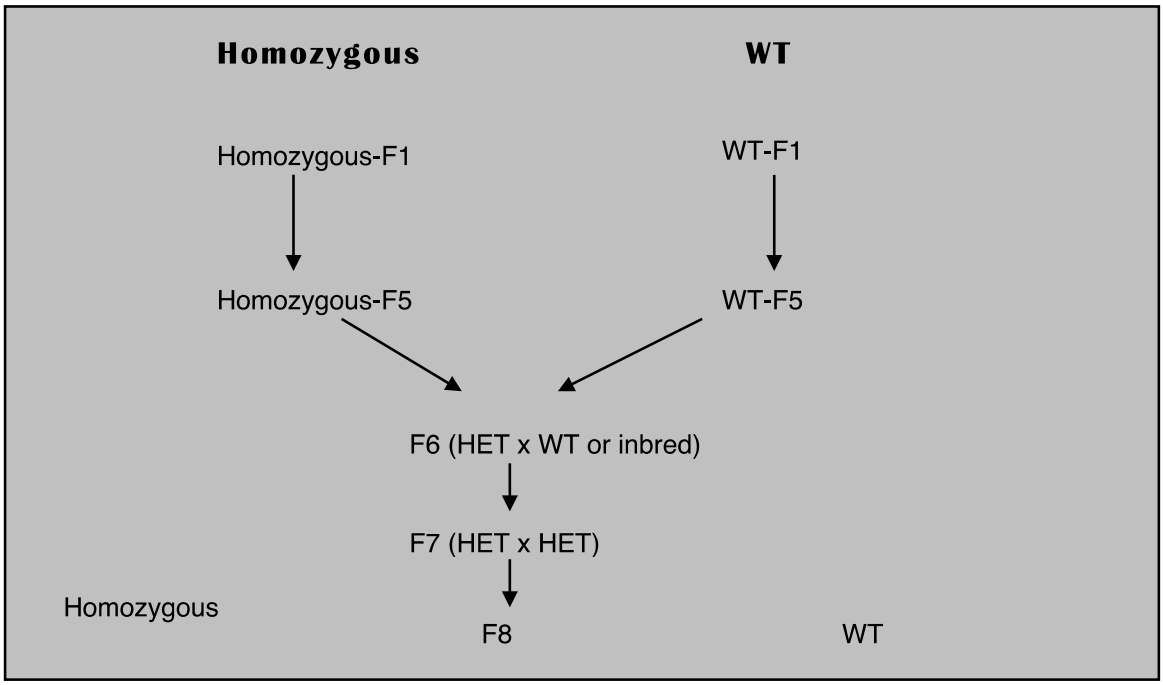
- Every 5 generations, lines that are being line bred as homozygotes need to be backcrossed to the background inbred strain to minimise genetic drift.
- For example, a researcher is running a homozygous knockout on a pure B6 background needs to cross the knockout line to a B6 line to ensure there is not significant genetic drift from the control line.
- If the knockout line is of a mixed background, the line should be backcrossed to WT controls or relevant F1 hybrids for the same reasons.
- One alternative breeding pattern is to maintain one heterozygous pair that can produce replacement homozygous breeders as well as WT controls for experiments. This pair is produced by mating homozygous mouse to a mouse from the background inbred strain. *See example 2 below*. Note: the heterozygous x heterozygous pair should not be left continuously breeding unless there is a large number of homozygous pairs to replace. (ie. > 6 pairs)

Controls for a pure line

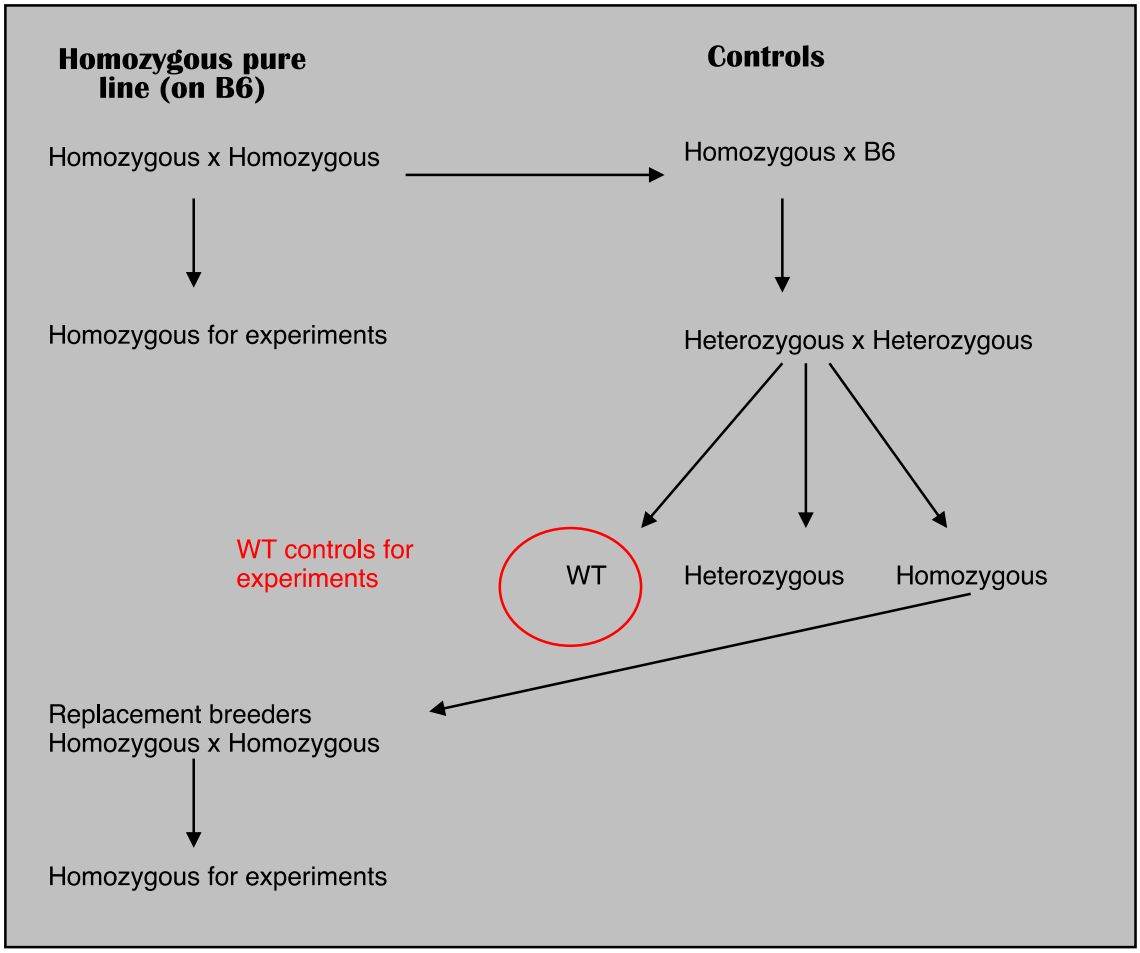
- If the breeding program is running a homozygous line that is fully backcrossed to an inbred strain (for example, B6), the control for these mice is an inbred B6 mouse.

- If heterozygous animals or WT animals are required, a B6 can be crossed with a homozygote to generate the required animals.
- A WT line should not be maintained long term as a separate line without any inter-crossing. This will result in 2 divergent lines, where the WT colony no longer represents a control.

Example 1 : 5th generation backcross



Example 2: breeding to provide WT for experiments and replacement breeders for homozygous pairs



Points for GM lines:

- Replacement (GM) breeders should always be genotyped, (even if they are from homozygous parents) or the first litter of each new breeding pair must be genotyped.
- A homozygous line should have a minimum of 2 breeding pairs with 4 boxes of reserves (2 boxes of females and 2 boxes of males).
- On the 4th litter, set aside replacement breeders, so by the 5th litter the breeding pair can be replaced.
- Pairs should not be culled until it has been established that the new breeding pair is 1) the correct genotype and 2) able to produce good litters of the correct genotype.
- If a litter is destroyed, it needs to be included on the production records.
- When the pairs are being set-up, the following steps need to be followed for each pair:
    - The date and pair number of the mouse on the stock card needs to be verified.
    - The breeding card needs to be checked. Do the dates, litter number, number of males and number of females correspond with that on the stock card?

Minimising over-production:
Overproduction must be avoided wherever possible to minimise wastage. Ways to minimise overproduction are:

- Keep minimal breeding pairs if only maintaining the line (see section- maintaining the line)
- Keep minimal nos. breeding pairs when backcrossing (see section – backcrossing)
- Ensure mice are genotyped promptly so that errors in breeding are detected as soon as possible
- Ensure homozygous lines are genotyped regularly
- Adjust the number of breeding pairs if supply exceeds usage.
- Ensure that animal husbandry activities minimise losses eg:
    - Minimise disturbance of litters and provide adequate nesting material
    - Wean on time so that newborn pups are not trampled by weaners
    - Check for flooded cages on a daily basis and check food and water is available
    - Mate and replace breeders at the optimal age
- Freeze down lines no longer being used.

Problem breeding

General

- Identifying problem breeders:
    - History (from production figures)
    - Published literature on the line may warn of phenotype problems

- Sometimes breeding starts to decline as a result of 'inbreeding depression' especially if brother / sister mating has been practiced. One way to combat this is to cross the line with the background inbred strain or cross the GM mice with their wild-type counterparts. Obviously the offspring need to be monitored/tested to ensure that they still carry the required phenotype and genotype.

Resurrecting a line

- If a line is lost it may be recovered from:
    - Cross lines carrying the gene
    - Line owned by another researcher
    - Frozen embryos/ sperm
    - Re-sourced from another institute

Monitoring

- Breeding performance (births, litter size, interval, mortality, sex ratio) is monitored via analysis of the production records.
- If there is an indication of spontaneous mutation which impacts on the animals eg malocclusion, seizures, overgrown teeth you will need to cull the breeding pair generating the mutation. Any of the pairs offspring, even if not affected, should not be used as replacement breeders.
- Any unexpected phenotypes or an increase in unexplained deaths need to be investigated.

Cull criteria

General guidelines are listed below however it must be noted that some lines breed poorly due to a phenotype impacting on reproductive performance and this will alter the relevance of the cull criteria. In some cases the mice breeding poorly may be homozygous and may produce the best progeny for replacement breeders.

- No litters within 2 months from the date of mating.
- Inter litter interval should not exceed 6-7 weeks without investigation.

- Destroyed litters are tolerated on the pair's first litter. Cull mice that cannibalise their first and second litter. If cannibalism is frequent other causes, such as environmental problems, must be investigated and recorded in the production figures.
- Litter size less than average for the strain/line of mice.
- Cull sexes not needed near weaning. Cull back excessively large litters especially if single sex is required.
- Cull runts or obviously sick animals.

Record keeping

- Daily check is required of EVERY box for food and water .
- Cage cards (breeding cards) should be archived.
- All breeding pairs must be carefully checked twice per week.
- See "Breeding- Production records" for record keeping requirements

Sentinels

A sentinel program operates within select LAS facilities.

- These are used to monitor possible contamination by the transfer of dirty bedding from the screened to the sentinel cage.
- The testing screens at the rack level, not the individual line level so it is important to avoid moving breeding boxes from rack to rack.

Cards

See LAS staff for appropriate breeding and stock cards.

The following are **examples** only to illustrate the type of information which should be recorded on a cage card.

Breeding Card

Once a male and female have been mated to produce live progeny, a breeding card must be attached to the breeding box. The breeding card stays with the female at all times.

<PAIR NO> <LINE NAME> **5 G6B6Δ**							BORN: **10/3/02** MATED: **10/5/02**		
	NO. BORN		NO. CULL		Mortality		NO. WEAN		COMMENTS
	<male# & genotype> <female# & genotype> **m G6B6Δ/+ . 66 X f WT . 99**								
DOB	M	F	M	F	M	F	M	F	
17/6/02	**3**	**4**	**3**			**1**	**0**	**3**	
15/7/02	**5**	**5**	**5**		**0**	**3**	**0**	**2**	
16/9/02	**0**								DL - cull
									Poor breeder

Stock Card

<Line Name> . <Pair number / Litter Number>	<Number of mice in the box/Sex of mice>	<Ethics number and researcher initials>
G6B6Δ . 10/1	**5 males**	**01/01 RH**
	b: <Date of birth>	**01/01/04**
	<TC or EM and date>	**TC 05/02/04**

<ID number>	<Genotype>
1	**G6B6Δ/+**
2	**WT**
3	**G6B6Δ/ G6B6Δ**
4	**WT**
5	**G6B6Δ/ G6B6Δ**

Each line needs a different card colour.

Housing of breeding stock

- No single housing unless justified i.e. studs that have been fighting or specific research procedures that require isolation may be adequate justification. If so the reason for single housing must be written on the cage card.
- Maximum stocking 5/box. But aged males should not be housed at >3/box.
- If studs must be retained and not used for more than 1 month then they need a partner mouse.
- If a harem system is used the females must be separated before the litters are born if both are pregnant. Two females with litters constitute overcrowding.
- Mice that have large litters (i.e. Swiss mice) may need an increased box change frequency.

Timed Mating

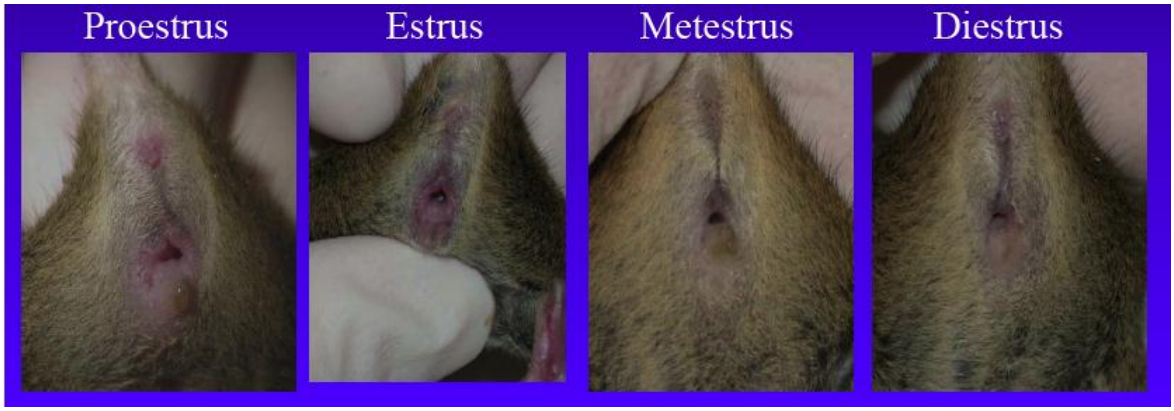
- Mice that are going to be time mated need to undergo Whitten affect for 3 days prior to pairing.
- Pair a male and female and record the date of pairing and the female's id number.
- Plug check the female the next morning and continue until she plugs positive. When the female has plugged, separate her from the male.
- Record the use of the female in the lab book. These mice will be required to be reported in the annual animal usage report to NSW government.
- If a specific plug date is required separate all females from the stud on the day of plug check.
- Note: the date of the plug check is considered Day 0 or day 0.5 of pregnancy.

Plug Checking Procedure

- Pair a female with a stud male as late as possible (afternoon or evening) before the lights are turned off.

- As early as possible the next morning, separate the female from the male and check for plugs before re-housing her. Lights should be on full strength before trying to see plugs.
- Plug check by placing female on wire lid (stud's box) pull female in a backwards motion with base of tail, she should pull forward with front legs.
- Raise her hindquarters so you can view the vaginal area (vagina is the lower opening - under anus).
- Using a stainless steel probe (round end), prod the opening to feel for a hard substance clogging the vaginal area, normally yellow/white in colour. The probe will not enter the opening at all and the plug is normally visible.
- Some plugs can be very difficult to see immediately. They can be clear, soft or sticky with residue present and can be very deep. So you need to place probe inside and lift upwards to open up the vagina.
- Separate females into boxes remembering to only house positive plugs together and negative plugs separately otherwise the oestrous cycle of the non pregnant mice can influence the positive plugs to return to oestrous and abort the pregnancy.

Oestrus checking (from 'Reproductive Biology of the Mouse – Rob Taft)
The state of oestrous in a mouse can be determined by the visual appearance of the vagina.



Whitten Effect

- The presence of only females in a cage may depress the oestrous cycle of all females in that cage. The addition of a male to the cage causes all such females to initiate oestrus in about three days. This phenomenon is called the Whitten Effect. Mouse breeders often take advantage of the Whitten effect to enhance timed pregnancies.
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- This effect is better noted in females that have been acclimatized to the environment and away from all males for a period of time. This will allow a large portion of the female mice to enter an anoestrus or quiescent state and this is known as the Lee-Boot Effect. When exposed to the urine, it is reported that at least 75% of mice will be in oestrus or pro-oestrus within 3-4 days (results are strain dependent). This effect is enhanced by a longer duration of isolation from male mice prior to exposure to urine.
- *Procedure*: Group house some female mice. Everyday for 3 days, place a small handful of bedding from a box of males in the box (the dirtier the better). Late on the 3rd day, pair the males and females. Plug check on the 4th day.

Test Mating

- Test mating is carried out to genotype animals when the researchers are unable to determine if a mouse is homozygous or heterozygous using molecular biological tools (Southern Blot or PCR).
- If a mouse is suspected of being homozygous or heterozygous for the gene of interest, it is crossed with an inbred wild type mouse. The progeny of this test mating are then examined. If there are wild type genotypes in the progeny then the parent mouse is a heterozygous. If the progeny are all positive for the gene, then the parent was a homozygous mouse.
- A minimum of 7 pups needs to be examined to feel confident that a wild type can be detected.. The normal procedure involves killing the neonates and placing the whole tail in an Eppendorf tube. These pups must be included in your annual animal usage returns.

NOTE: The breeding pair needs to be separated as soon as the female is observed to be pregnant. They may need to be re-paired at a later date if not enough pups are produced in the initial litter.

- The details of the pairings and progeny need to be recorded.

Cages

- Boxes for a particular line should be kept together in a room (i.e. On the same rack).
- Ideally the boxes should be organised so that the mice are in numerical order.
- Space should be allocated on the same rack for the weaners. For example, if *on average* the line produces 3 boxes of weaners, space for 3 boxes should be allocated.

Crossed lines/Experimental lines

- What is the procedure for new lines?
- Compliance with LAS entry procedures is required.
- If heterozygous lines are to be bred, it is expected the genotyping protocol has been established.
- If LAS staff are to perform the breeding, a breeding plan must be discussed with the LAS operations manager.
- If the breeding is to be conducted by the research group you will need to discuss space allocation and provide a copy of the phenotype report.
- LAS technicians will watch for new phenotypes or changes in the mortality of the mice and report these to the investigator if they are managing the breeding colonies.

Nomenclature

- See the *Nomenclature* guidelines on the Jackson Laboratory web site (www.jax.org). The correct nomenclature should be used in the phenotype report but abbreviations can be used on the cage cards.

Examples of some common abbreviations

Name	Abbreviation	Name	Abbreviation
SV129/J	129	SJL	J
C57BL/6	B6	FVB/n	FB
DBA	D		
BALB/c	C		
Swiss	QS		
SVEV	EV		

Breeding specific genotypes

- If breeding toward a particular goal is required – for example, generating knockout mice from heterozygote crosses or trying to generate double knockout mice by crossing 2 homozygous knockouts.
- Aside from any phenotypic factors that may result from the missing gene(s), there is an expected ratio of genotypes generated. This must be considered in light of the number of mice that are a required to generate a new line or to produce a mouse with a specific genotype

See Attachment 4 for breeding probabilities.

Backcrossing Lines

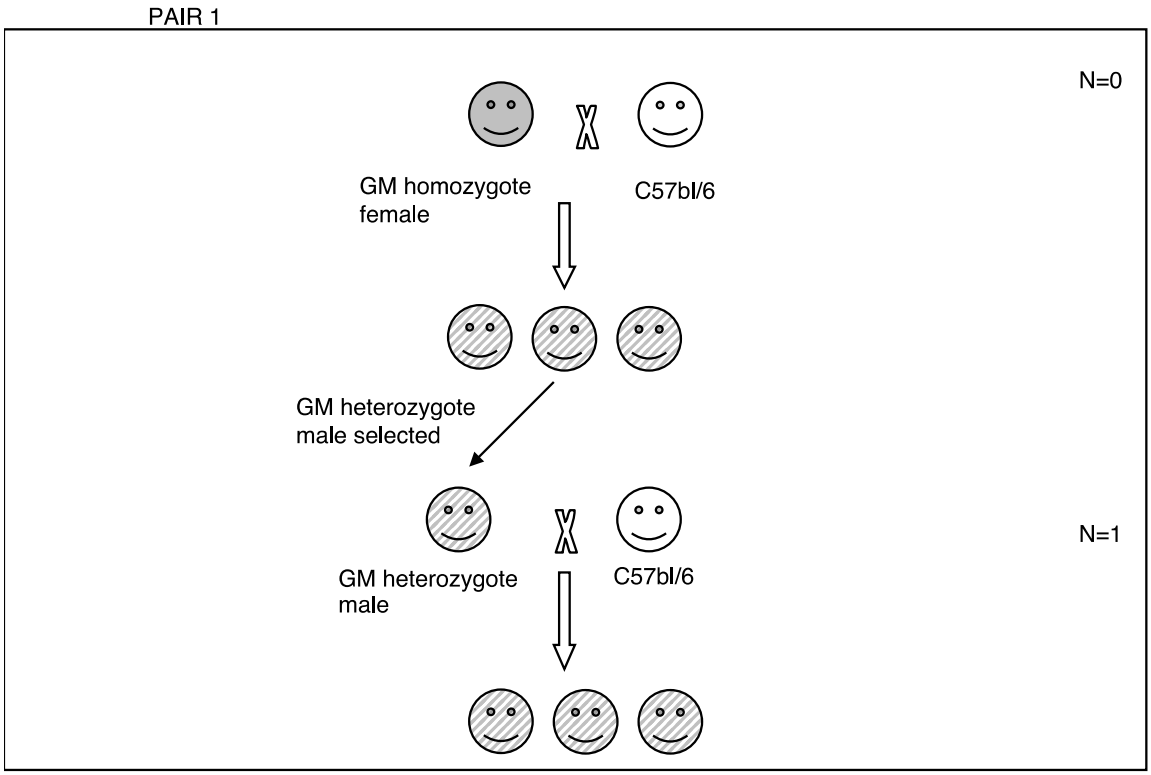
Regular Backcross

- Should be backcrossed onto an inbred strain for a minimum of 10 generations as per the normal protocol.
- At least 2 pairs should be running concurrently during a backcross. This prevents any issues, should there be a problem with one of the crosses (e.g. mutations).
- Males and female GM animals can be crossed with the inbred strain. In order to promote variability, males and females can be alternated. If a male GM is crossed with an inbred strain, one of it's female homozygote progeny should be taken for the next cross. *See attachment 3.*
- After 3 generations, 2 GM heterozygotes should be put together to assess the impact of the backcrossing. This exposes any mutations that might have developed. It's better to find out at N=3, rather than N=10.
- At least 3 generations of the backcross should be present at any one time. When the progeny of N=3 **are weaned**, the first generation can be killed.

Generation Number

The first pair set up to initiate the backcross is called N_0. The progeny of this pair becomes N_1. See the diagram below

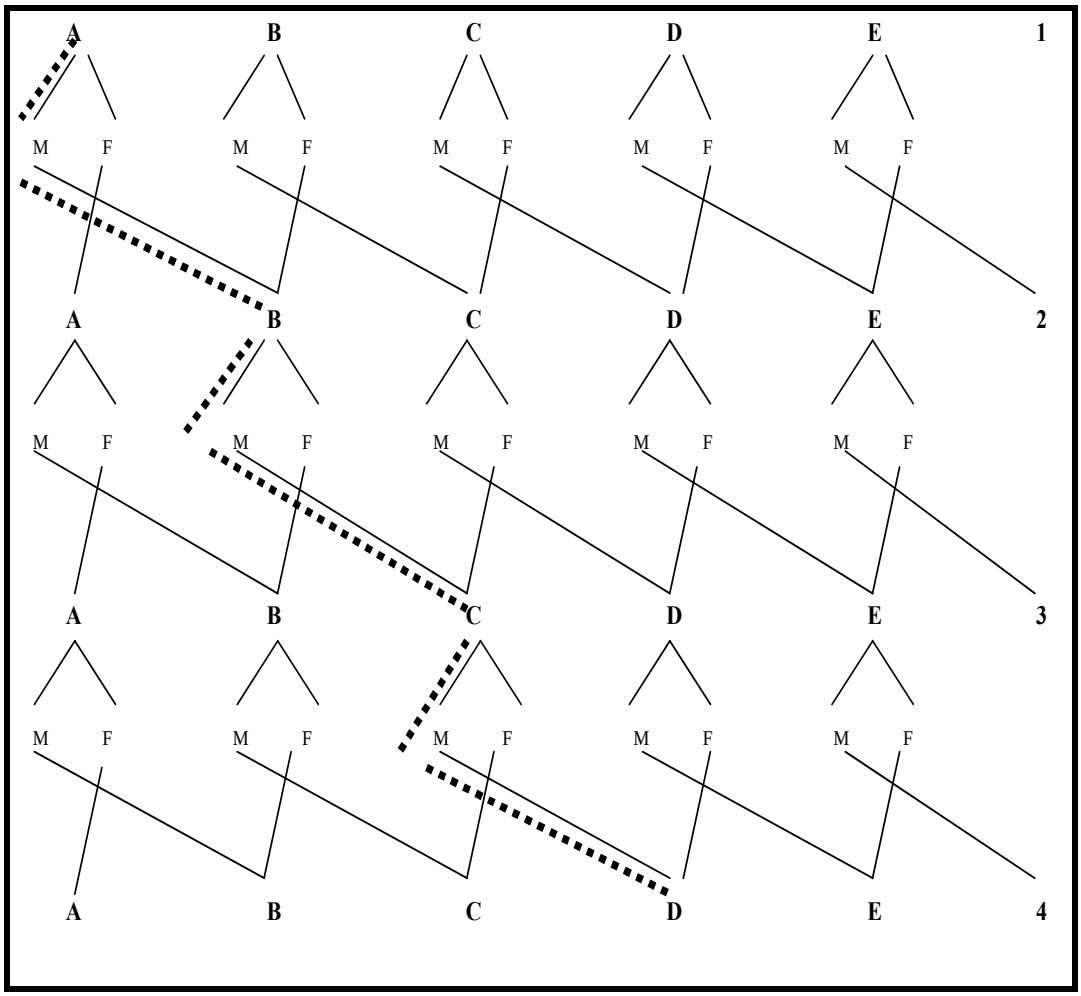
Backcross Protocol


PAIR 1
N=0
GM homozygote
female
C57bl/6
GM heterozygote
male selected
GM heterozygote
male
C57bl/6
N=1

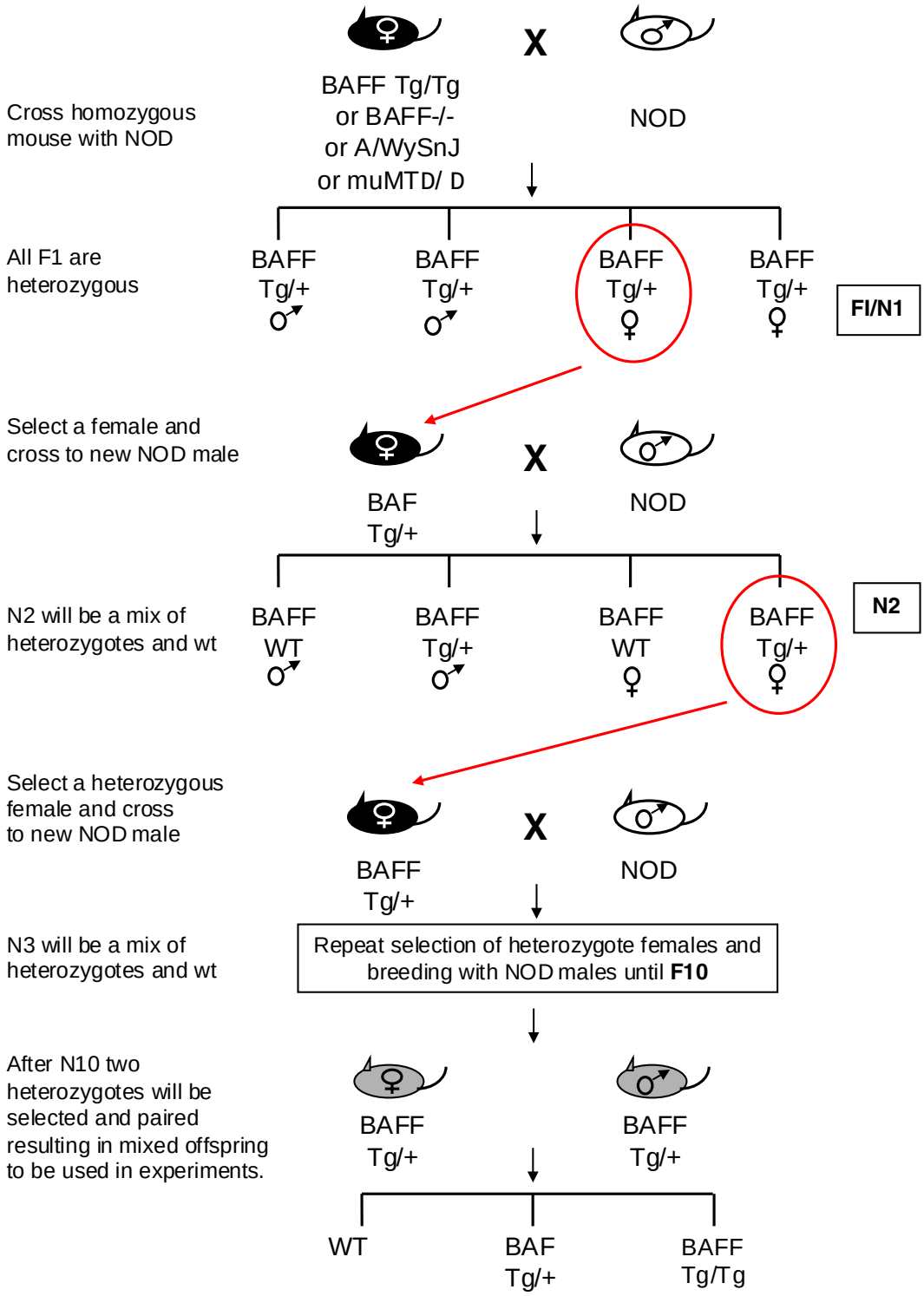
Outbreeding Scheme

Outbreeding Qs Colony
Controlled Minimal Inbreeding (outbred)

The colony is divided into a number of groups which should be roughly equal in numbers and past breeding performance. The more groups the colony is divided into the more generations that are involved before the program returns to the beginning. Usually the colony is divided into four to eight groups which are labelled alphabetically.



NOD Backcrossing



Genotype Probabilities

*Crossing PΔ/+ **x** PΔ/+*

	PΔ	+
PΔ	PΔ/PΔ	PΔ/+
+	+/PΔ	+/+

25% PΔ/PΔ
25% +/+
50% PΔ/+

*Crossing PΔ/+ YΔ/+ **x** PΔ/+ YΔ/+*

	PΔ	+
PΔ	PΔ/PΔ	PΔ/+
+	+/PΔ	+/+

	YΔ	+
YΔ	YΔ/YΔ	YΔ/+
+	+/YΔ	+/+

PΔ/PΔ YΔ/YΔ	**1/4 x 1/4**	**=1/16**	**(6.25%)**
PΔ/PΔ YΔ/+	1/4 x 1/2	= 1/8	(12.5%)
PΔ/PΔ +/+	1/4 x 1/4	=1/16	(6.25%)
PΔ/+ YΔ/YΔ	1/2 x 1/4	= 1/8	(12.5%)
PΔ/+ YΔ/+	1/2 x 1/2	= 1/4	(25%)
PΔ/+ +/+	1/2 x 1/4	= 1/8	(12.5%)
+/+ YΔ/YΔ	1/4 x 1/4	=1/16	(6.25%)
+/+ YΔ/+	1/4 x 1/2	= 1/8	(12.5%)
+/+ +/+	1/4 x 1/4	=1/16	(6.25%)

The cross above can also be expressed as percentages

Double Knockouts-Pt2

	C2Δ/C2Δ	**C2Δ/+**	**+/C2Δ**	**WT**
D3Δ/D3Δ	D3Δ/D3Δ C2Δ/C2Δ	D3Δ/D3Δ C2Δ/+	D3Δ/D3Δ +/C2Δ	D3Δ/D3Δ WT
D3Δ/+	D3Δ/+ C2Δ/C2Δ	D3Δ/+ C2Δ/+	D3Δ/+ +/C2Δ	D3Δ/+ WT
+/D3Δ	+/D3Δ C2Δ/C2Δ	+/D3Δ C2Δ/+	+/D3Δ +/C2Δ	+/D3Δ WT
WT	WT C2Δ/C2Δ	WT C2Δ/+	WT +/C2Δ	WT WT

This table describes the range of genotypes that can result from crossing 2 double heterozygous animals. There are 16 possible genotypes (in grey). For example, the chance of a double homozygous animal is 1/16. This is represented by the presence of one square. In contrast, the possibility of obtaining double heterozygote animals is 4/16 or 1/4.

Pedigree Chart