

# Cryopreservation Advisory for Mouse Strains used in Medical Research

September 2012

## 1. Purpose

This advisory is written for those who would like to understand the processes of mouse strain cryopreservation, why it is important and how to take advantage of the services offered. The most efficient method to protect mouse strains is by cryopreservation of gametes or embryos. Cryopreservation, and especially re-animation from frozen material, is not a trivial exercise, nor is the establishment of a cryopreservation and IVF laboratory cheap. There are numerous laboratories around the world that offer these services. Australia is well serviced by several laboratories working under the umbrella of the Australia Phenome Bank. Researchers should take advantage of the services offered and ensure their strains are archived safely and as soon as mice of the correct genotype are available.

### *Advisory Summary – Some of the questions considered:*

- Why should murine strains be cryopreserved?
- When should strains be cryopreserved?
- Should sperm or embryos be cryopreserved?
- What are the optimal storage conditions?
- How are frozen stocks re-animated?
- How long does it take to get a strain back from the frozen archive?
- The availability of databases and repositories.
- What advances in technique have been made?

## 2. Why your murine strains should be cryopreserved

Genetically modified (GM) mouse strains are vital to biomedical research. Hundreds of thousands of dollars go into creating and characterising each new strain and successful grant applications and manuscript preparation depend upon reliable access to these strains. For these reasons genetically modified strains need to be safe guarded against factors that threaten their livelihood and their availability. Once a GM strain has been lost the costs to start all over again may be beyond some research capabilities, with the possibility of some mouse strains not able to be produced again.

An efficient method for protecting these valuable resources is to cryopreserve the strain as gametes or embryos and store at temperatures preventing metabolic activity.

The main advantages are:

- Insurance against loss
- Save money

### 2.1. Insurance against loss

As stated above, one very important reason for cryopreserving GM strains is one of insurance against a complete loss of the strain. There are both external and internal threats to each and every colony.

#### 2.1.1. External threats

Housed in a purpose built animal facility one could assume the animals are safe from external disasters. However, natural disasters such as flooding, earthquakes, fire could harm an animal facility. Indeed, the animal facility at the University of Queensland was within metres of being flooded in January 2011. Flooding in Houston in 2001, resulted in the loss of 100,000 animals bred for research purposes. In February 2007 the ANU campus was hit by a freak ice storm resulting in major damage to over 60 buildings, including one of the animal holding buildings.



Despite animal houses being controlled environments, adverse weather

conditions including humidity, smoke from bushfires, extreme cold and heat still affect colony breeding and animal behaviour.

#### 2.1.2. Internal Threats

There are many internal threats to the GM colony. These include equipment and building failure, human error, and breach of biosecurity (i.e. infection).

##### 2.1.2.1. Human Error

The biggest risk to a mouse colony is human error. Unintentionally, the viability of a strain can be impacted by many errors involving humans. These include:

- Forgetting to refresh the breeding pairs during a period when animals are not required for experimental purposes,
- Inappropriate colony management (breeding) plan enacted,
- Genotyping errors, resulting in incorrect mice used as breeders,
- Identification errors leading to the incorrect mice used as breeders,

- Staff changes leading to unfamiliarity of the strain nuances.

#### 2.1.2.2. Pathogens

Pathogen infection can influence a colony viability by:-

- Resulting in poor breeding, causing the strain to reach critically low numbers,
- Phenotype of mouse is compromised, thereby rendering the mice unsuitable for experimental use,
- Sick animals need to be culled.

#### 2.1.2.3. Breeding

Changes in breeding rates can have drastic effects on colony maintenance. Breeding rates can be affected by:

- Breeding /reserve breeding stocks have become too old to breed,
- Environmental factors within the facility/room/rack effect the colony's breeding eg building works, staff changes, bedding, light, humidity, switch in cage design,
- Turn around of genotyping results is too long,
- Too few animals of correct genotype available resulting in an increased risk of insufficient breeder stock.

## **2.2. *Save money***

Importantly, cryopreservation of strains allows the deletion of live mice from the shelf when not required for experimental work, freeing up animal facility shelf space, and saving money required to maintain a line as a "tick over colony". The Jackson Laboratories have estimated it costs approximately \$10,000 a year to maintain a colony in "tick over" mode when space, time, genotyping, agistment and staff are taken into consideration. Cryopreservation allows the strain to be available in the future to take advantage of technical and knowledge breakthroughs.

### **2.3. The cost of the loss**

Does it really matter if the strain is lost? Consider the possibilities discussed in Scenario 1.

Scenario 1:

Your research team have identified a genetic modification that alters a biological pathway. The next step is to generate a mouse line expressing this mutation. The task of generating the line is outsourced to a laboratory / facility that specialise in generating genetically modified mouse strains. The fee maybe \$50,000 to \$90,000 and a minimum of 9 months are required to provide mice to establish a working colony.

A post-doc and an RA are employed to characterise the mutant strain using funds obtained from a Federal Government funding agency. The grant is worth \$500,000 over three years. Things are going well but then while the RA is on leave the post-doc takes over managing the colony and makes a mistake. By the time it is realised the wrong mice were set up for the next breeder pair, there are no mice left carrying the mutation. Line gone, research not finished. Now you need to spend more money regenerating the mutant, possibly another \$30,000<sup>1</sup>. Worse, the grant is now supporting 2 salaries but not producing any results and at least 6 months of the grant period are no longer productive. This is an obvious waste of public funds as well as laboratory mice from an animal welfare perspective.

All of this waste and loss of mice for critical experiments can be avoided by setting aside 2 - 5 male mice carrying the desired genotype and submitting the mice to a cryopreservation laboratory for a small fee. The Australian Phenome Bank can cryopreserve sperm from your strains.

### **3. When should your strains be cryopreserved?**

As soon as the strain has been established, several males of the correct genotype should be put aside as sperm donors. The reasons are two fold.

As stated above, once frozen down the strain is protected from disaster.

Secondly, early preservation of the strain protects against genetic drift. With each generation, random changes accumulate in the mouse genome. These changes may be silent or may have an effect on phenotype. Freezing down gametes or embryos as close as possible to the time of strain generation allows one to re-animate from the frozen stock and return to the original genetic state, providing increased confidence the strain you are working with now is the same strain you started with.

Unfortunately, the common trend is to cryopreserve a strain once all the experimental work has been completed. While this may be more convenient as all the mice of the desired genotype can be used for experiments, the strain is not protected against loss or genetic drift.

In addition, due to high cage costs there is a tendency to keep breeder and stock cages at the minimum. Minimising the number of “stand-by” mice may place the colony under breeding pressure. There is a tendency to “milk” a breeder pair for as long as possible.

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<sup>1</sup> Less than set up cost as ES cell clones are already established from first attempt.

As the mice age, or as the influence of the genetic modification becomes more apparent, the fertility of the breeder pair may decline rapidly. It is all too common for the one and only breeder pair to stop producing litters. Without frozen back ups, the strain is precariously close to being lost altogether.

Many a researcher has called upon the services of an experienced IVF laboratory to rescue a strain using the one remaining, aging and “inactive” male. It may already be too late. If sperm are present but dead, ICSI can be used to fertilise oocytes (see below), but if sperm production has been shut down altogether then there is not much that can be done. Worse than just aging, the one critical mouse maybe sick. Our observation has been sperm production is shut down in unwell mice and by the time the animal care staff notice the mouse is unwell, there maybe no sperm remaining.

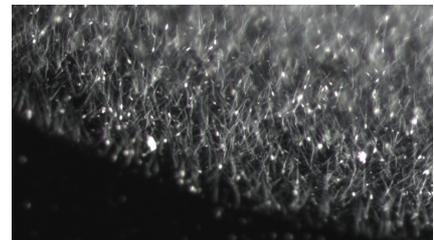
#### 4. Should you cryopreserve sperm or embryos?

The APB offers subsidised sperm and embryo cryopreservation. Sperm cryopreservation is conducted at The Australian National University, Monash University and the Animal Resources Centre in Perth. Embryo cryopreservation is conducted at the Animal Resources Centre. The embryo cryopreservation subsidy is only granted if the strain cannot be cryopreserved in the sperm format. The subsidies can be provided due to the generous support of the Australian Federal Government through several agencies.

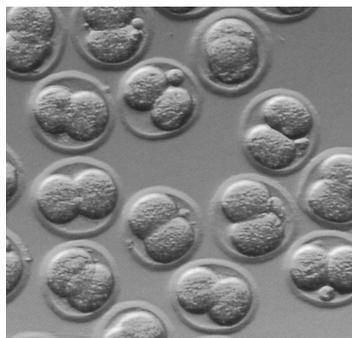
The most important criteria for deciding upon sperm or embryos are:

- Costs associated with the procedure,
- Costs associated with breeding the donor animals,
- Costs associated with re-animation (see table 1 for comparison),
- Sex chromosome linked lethality or fertility,
- Time required from re-animation to working colony,
- Effects of the mutation on male and female fertility.

Cryopreservation of sperm is by far the cheapest and easiest option. The APB will cryopreserve sperm from 5 males. The sperm is divided into ten aliquots, providing multiple opportunities for strain reanimation.



The major advantage of cryopreserving embryos is the conservation of the entire genetic make-up of the strain. When the embryos are thawed, transferred and the resulting pups are born, the genetic make-up of the pups is exactly the same as the embryo donor it was derived from. It is generally accepted 250-500 embryos should be cryopreserved for each strain. Since each female donor can at best provide 20-25 embryos, a lot of breeding is required to generate the donors, resulting in high cage costs. While the APB covers the costs of cryopreserving the embryos the researcher must cover the cage costs. For strains with poor breeding performances, the time required to store 500 embryos may be more than 1 year and the associated cage costs may total more than \$10,000<sup>2</sup>.



<sup>2</sup> The Jackson Laboratories “tick-over” estimate (see 2.2).

#### **4.1. Can you have confidence in the cryo-archive?**

While aliquots of sperm can be thawed and subjectively examined either manually or by computer assisted methods, the test thaw only indicates whether the sperm are alive and motile. Observation of motile sperm in the test thaw does not guarantee the sperm are capable of fertilising oocytes, nor whether live animals can be derived thereafter. To be more confident, IVF should be conducted and the resulting 2-cell embryos transferred into recipient females and left until pups are born. Further confidence can be gained by genotyping the resulting pups. The APB conducts the subjective test thaw as part of the cryopreservation process. Researchers can request IVF and transfers are conducted for a fee of \$550.

While the APB does not have the resources to include an IVF test for each strain submitted for sperm cryopreservation, IVF is performed regularly using samples chosen randomly from the archive as part of the quality assurance program. This program ensures the high standards of sample collection, sample maintenance and live born recovery are maintained.

Likewise, an aliquot of frozen embryos should be thawed and transferred into recipients and the number of pups born recorded.

Although test thaws and observation of live pups born recorded it is not possible to provide 100% guarantee a cryopreserved strain can be recovered from the archive.

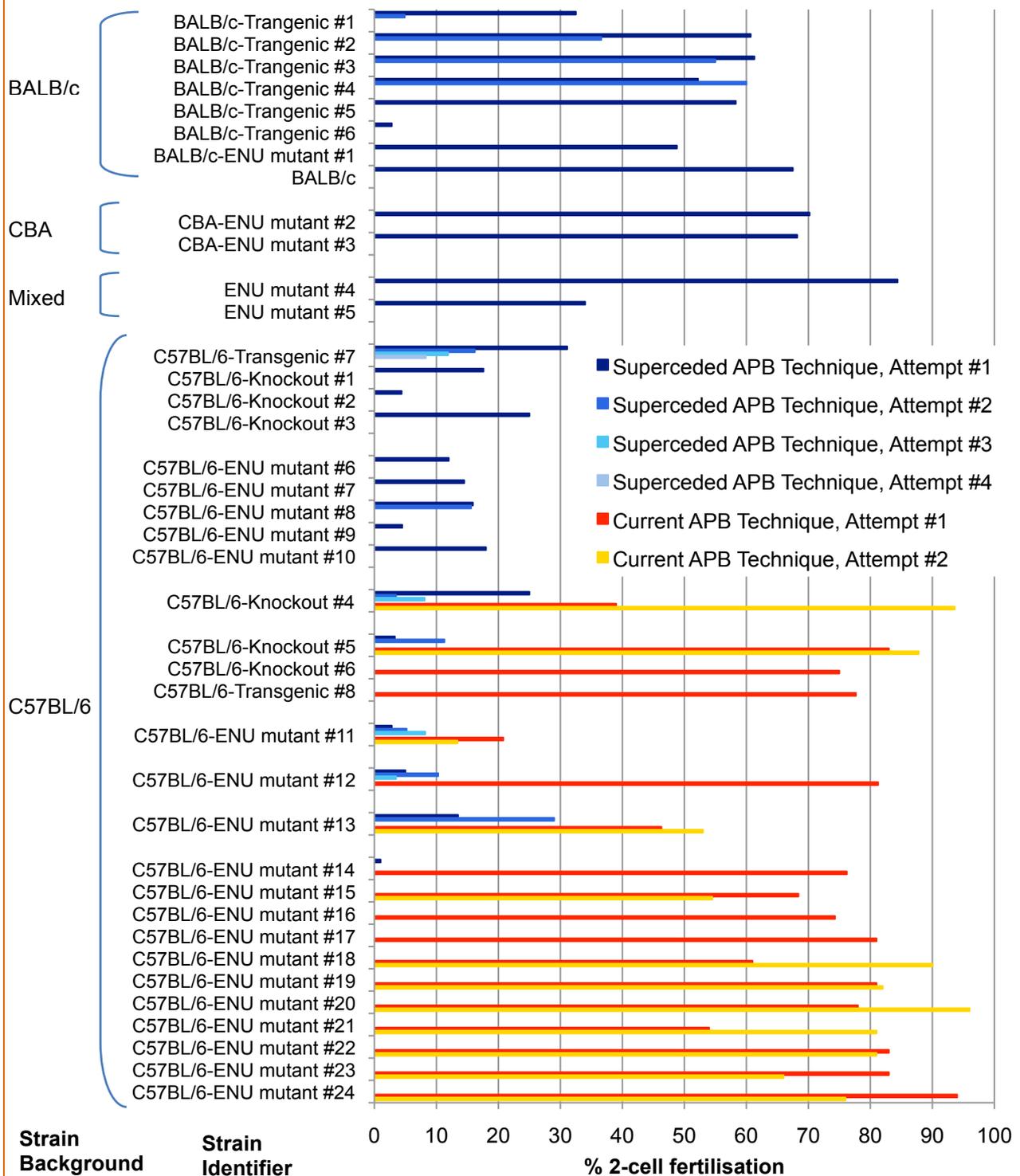
Success of strain re-animation from frozen stock is dependent upon the strain background. Recent technical advances have increased the fertilisation rate of frozen/thawed sperm from C57BL/6 background mice from 10 – 40% to 70-100% (see appendix 1 for explanation).

The APB has achieved similar rates of fertilisation using procedures including the technical advancements (see Figure 1). Using the old (superceded) technique, APB staff achieved up to 60% fertilisation with strains on the BALB/c background and greater than 60% for strains on CBA and mixed background. As mentioned sperm from C57BL/6 background strains does not freeze well and the fertilisation rates of the thawed sperm is usually very low. At the APB these rates were 0-20%.

Adoption of the Nakagata modifications (current APB technique) has resulted in 2-cell fertilisation rates for strains on C57BL/6 background increasing to 70-90% (ave = 71%, range: 13.4 – 96%, compared with average of 12% for the old technique). Even with the new technique there is still strain-to-strain variation in fertilisation rates. Live pups were recovered from the strain that had the 13 and 20% 2-cell fertilisation rate.

Please note the APB staff do not see a correlation between the 2-cell fertilisation rates and number of pups born after the 2-cell embryos are transferred into recipient females.

**Figure 1:** Comparison of 2-cell fertilisation rates for IVF performed by the APB using the superceded and current techniques.



## 4.2. Considerations

- While sperm homozygous for the genetic manipulation may be stored, during the IVF procedure the sperm must be incubated with oocytes that do not carry the modification. Thus, at best, the animals produced from the IVF will be heterozygous. Depending on the experimental requirements another generation of breeding may be required before animals will be available to use.
- Sperm concentration, motility and activity can vary from mouse to mouse, even within litter siblings. It is thus advised sperm from more than one animal is stored. While the sperm from several individuals can be pooled before aliquoting, the general practice is to not pool.
- Age of the donor: Sperm donors need to be a minimum of 10 weeks of age. Fertilisation results are significantly diminished using sperm collected from mice younger than 10 weeks. As the mice age beyond 20 weeks the sperm concentration and viability may decline, again affecting IVF efficiencies.
- Sick mice tend to shut down sperm production, mice should preferably be of optimal health at the time of sperm harvest.
- Genetic modifications can affect fertility. For some strains sperm cryopreservation is therefore not a viable option.
- The techniques used by most laboratories around the world involve the euthanasia of the male before harvesting the sperm. If tissue samples need to be collected at the time of death, sperm collection must be processed first to minimise sperm death.

**Table 1:** Summary of considerations for choosing sperm or embryo cryopreservation

Consideration	Sperm	Embryos
Time required	Quick (hours)	Potentially a long time (months)
Number of animals	5 males	Colony (possibly for 1 year)
Age of Donors	Age 10 – 26 weeks	7 – 9 weeks
Numbers Stored	11 straws / vials from each male (millions per straw)	400 – 600 embryos (20-25 per straw)
Cost <sup>3</sup>	Procedure: \$300-\$500 Cage costs <sup>4</sup> : >\$70	Procedure: >\$2,000 Cage costs: ~\$2,000
Testing	Test thaw (subjective) Thaw, IVF to blastocyst or transfer to achieve pups	Thaw and transfer. Higher confidence.
Re-animation	Thaw, IVF, embryo transfer. Cost: \$500 – \$2,000	Thaw, transfer. Cost: \$400 - \$1,000
Genotype / Phenotype	Only preserves half of the genetic make-up. At best resulting animals are heterozygous	Exactly the same as when freeze conducted.
Time to working colony	Possibly multiple generations	Immediate if homozygotes were cryopreserved

<sup>3</sup> Assumes established cryopreservation and IVF laboratory / facility used

<sup>4</sup> Assumes 1 cage of 5 males, \$10/week, age 10 weeks

## 5. Storage

### 5.1. Temperature and storage vessels

To ensure long-term viability both cryopreserved sperm and embryos must be stored and transported at temperatures below  $-155^{\circ}\text{C}$ . Viable gametes have been recovered from samples stored below  $-155^{\circ}\text{C}$  for greater than 10 years.

Storage vessels cooled by liquid nitrogen are the preferred units to maintain the ultra-cold temperatures required for gamete storage. The APB stores all of the sperm and embryo samples in vapour phase liquid nitrogen cooled vessels. Since the liquid nitrogen is stored in the walls or the floor of the vessel, the risk of ultra-cold burns is reduced.



In addition, since the samples are not surrounded by liquid it is not possible for pathogens that may infect one sample to transfer to another. For this reason, the vapour phase units are the only units to be approved for use by BioSecurity Australia (formally AQIS) for the storage of imported frozen material.

All of the storage vessels used by the APB are housed in secure locations and are monitored by building management systems such that any equipment failure can be addressed quickly, greatly reducing the likelihood of the samples being destroyed.

### 5.2. Back ups

It is internationally recognised all samples should be stored in duplicate. That is, half of the aliquots for any one sperm or embryo sample should be stored in separate units. In addition, the second storage unit must be housed in a separate building. Thus, if one storage unit fails or is damaged and all of the samples destroyed a complete back up of the strains is available. The APB stores half of the samples derived from each mouse at The Australian National University and the other half at Monash University.

### 5.3. How many samples?

Assuming the sperm or embryos cope with the cryopreservation process and are capable of generation live pups of the expected genotype, then the number of samples stored for each depends on both the format of storage and then number of times the strain is going to be re-animated from the frozen stock.



For strains archived in the sperm format, theoretically, sperm harvested from one individual is enough. The sperm can be aliquoted into 10-20 straws or vials implying there are 10-20 options for strain re-

animation by IVF. However, to safely archive the strain it is recommended sperm be harvested from at least 2 and preferably 3-5 animals.

The APB recommends sperm is harvested and stored from 5 animals.

For strains archived in the embryo format, several factors must be considered. For the majority of cases, less than 25 embryos can be harvested from one donor female. Usually 10-15 thawed embryos are transferred into recipient females and not all of the embryos will survive to produce live pups. In addition, embryos are usually stored in aliquots of 20-25. Thus, embryos harvested from one female will at most be stored as one sample providing only one attempt at re-animation. It is therefore recommended greater than 100 embryos are stored for each strain. If the genetic modification results in poor fertility when in the homozygous state it is recommended a greater number of embryos are stored, preferably 500.

## 6. Re-animation from frozen stock

Re-animation of strains from frozen stock is a specialised exercise and is best performed by established laboratories, staffed by trained technicians with access to surgical and anaesthesia equipment.

### 6.1. Embryos

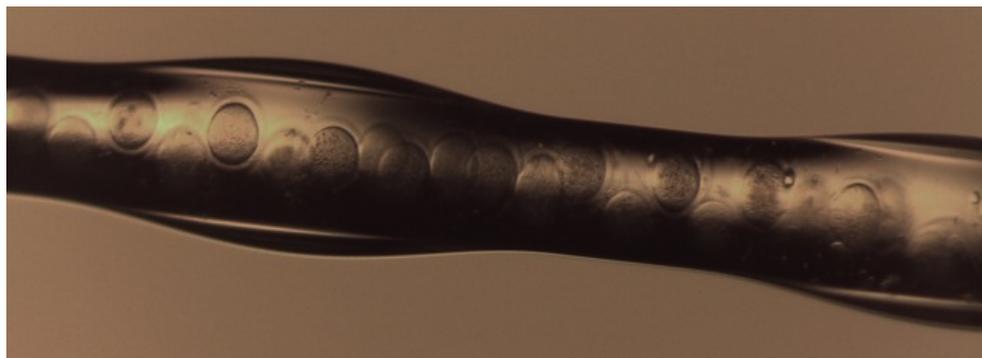
The process of recovering strains from frozen embryos is simpler than for sperm. The process involves thawing the embryos using an appropriate technique (the freezing method needs to be considered, see above). Thawed embryos are usually cultured overnight and then transferred into pseudopregnant females by surgery.

### 6.2. Sperm

Since sperm only carry half of the genetic template required for an embryo to develop, the remaining genetic material must be sourced from strain background matched oocytes.

#### 6.2.1. *In vitro* Fertilisation (IVF)

*In vitro* fertilisation involves the incubation of capacitated sperm with mature oocytes in a tissue culture dish. Briefly, sperm samples are recovered from the liquid nitrogen storage vessel, thawed and incubated for at least 30mins to regain “activity”, known as capacitation. During this incubation step, oocytes are harvested from female mice. (Oocytes can also be cryopreserved, and it is possible to thaw previously harvested oocytes and use these for the IVF). Once active, an aliquot of the sperm are introduced to the oocytes and left to incubate for 3 to 5 hours. After this time, the oocytes are washed, to remove all traces of sperm and then incubated



overnight at 37°C, 5% CO<sub>2</sub>. Successful fertilisation is determined by the presence of 2-cell embryos. The 2-cell embryos are then washed and transferred into pseudopregnant females. Pups are expected 20 days later.

### 6.2.2. Intra-cytoplasmic Sperm Injection (ICSI)

For a variety of reasons, including genetic modification and / or strain background, the sperm may not tolerate the cryopreservation process, resulting in reduced motility and possibly death. Under these circumstances, the sperm will not be able to “swim” toward the oocytes and / or be capable of penetrating the zona pellucida (the outer membrane of the oocyte). All is not lost if the sperm are incapable of fertilising the oocytes. In these cases, a technique termed intra-cytoplasmic sperm injection can be used. ICSI involves the injection of a single sperm directly into the cytoplasm of an oocyte using an injection pipette.

ICSI requires only one sperm per oocyte for fertilisation. The sperm do not need to be motile or alive for the ICSI process to be successful. Indeed, for mouse sperm, the tail is cut away from the head of the sperm and only the head is injected into the oocyte. ICSI is successful so long as the sperm nuclei are intact and the genetic material is undamaged.

Unfortunately, due to the costs of establishing an ICSI capable laboratory (requiring \$100,000 worth of equipment) and the requirement for highly trained and skilled technicians, not all IVF laboratories offer an ICSI service.

## 7. How long does it take to get my strain back from the frozen archive?

While the techniques for thawing and transferring embryos and for thawing sperm, performing IVF and transferring the resulting embryos can be conducted within 1 working week this does not mean the mice are available 7 days after a request for the strain be re-animated was received. Obviously, taking into account mouse gestation period, pups will not be born until 20-21 days post the embryo transfer day. Another 21 days must pass before the pups can be weaned, maybe longer for some strains that take longer to mature. Thus, if everything runs to plan the earliest mice would be available would be 6 - 7 weeks.

However, there are many other factors to consider that will / may affect how long it will take to get to a working colony. These include:

- Size of the cryopreservation and IVF facility operation. All of the facilities in Australia operate with a small staff number. As more and more strains become available, and more and more strains are archived, the larger the workload for each facility and the longer it takes to process each request. All requests are scheduled in order of receipt of the completed paperwork.
- Availability of mice. To generate pups from both sperm and embryo formats requires the transfer of the embryos into pseudo-pregnant females. Pseudo-pregnant females are generated by housing females, in estrous, with vasectomised males for 1 – 3 days. Females will only be in a pseudo-pregnant state after mating with the male.

- As mentioned previously, IVF using thawed sperm requires harvesting oocytes from 3-4 week donor females. These females must be ordered from the production colony usually 4-8 weeks in advance.
- Fertilisation rates vary dramatically from strain to strain. Even with the availability of improved IVF procedures, several IVF sessions may be required to produce enough animals to start a working, experimental colony.
- Even if 2-cell embryos are achieved after IVF, not all embryos will develop *in-utero*. The percentage of pups born compared to embryos transferred can be anywhere from 0 - 100%.
- Pups may die before weaning.
- Delays by the requesting institution to provide approval for shipment.

Taking all of these considerations into account the major repositories and facilities offering IVF service list the delivery time of 2-3 breeding pairs to the requesting client (after the receipt of all completed paperwork) to be 3 - 6 months.

## 8. Repositories

Almost a century of mouse research has led to the accumulation of large numbers of mouse strains – inbred, congenic, and spontaneous mutation strains, and strain panels. The advent of transgenic and gene targeting technologies, coupled with large scale mutagenesis and gene trap programs has led to a virtual explosion in the production and use of mutant and genetically engineered mice.

Large scale production programs such as the International Knockout Mouse Consortium (IKMC) are well on the way to generating knockout mutations for all mouse genes in embryonic stem (ES) cell lines, many of which are being used to produce novel mouse strains as the next step of the KOMP project (KOMP<sup>2</sup>) gets underway. Potentially, greater than 20,000 new mouse strains will become available in the next few years.

Centralized repositories are essential if these valuable resources are to be securely preserved and fully exploited. The establishment of centralized mouse repositories around the world for distributing and archiving these resources has provided critical access to and preservation of these strains.

For investigators, repositories secure against loss of their strains, enable efficient and cost-effective colony management, reduce the number of mice used in research (consistent with the 3Rs – replacement, refinement and reduction), relieve them of using research dollars and interrupting research to distribute mice, help meet the NIH and Wellcome Trust requirements for sharing research resources, and enable them to access a broad variety of strains and mutations for a variety of genes.

Rather than wasting funds duplicating the efforts of these repositories, researchers and institutions are better off utilising these repositories to cryopreserve, archive and distribute their mouse strains.

The major repositories include:

- The Australian Phenome Bank (APB): <http://pb.apf.edu.au/phenbank>
- European Mouse Mutant Archive (EMMA): <http://www.emmanet.org>
- Mutant Mouse Regional Resources Centers (MMRRC): <http://www.mmrrc.org/index.html>
- The Jackson Laboratories: <http://www.jax.org>
- Canadian Mouse Mutant Repository (CMMR): <http://www.cmmr.ca/index.html>
- RIKEN BioResource Center: <http://www.brc.riken.jp/lab/animal/en/>

The APB has established excellent working relationships with staff at each of the repositories listed. Technical information, assistance and advancements, frozen and live strains are regularly exchanged.

### **8.1. Access agreements**

Deposition of the genetically modified mouse strain into the archives maintained by the large international repositories does not mean the researcher or institution loses control of the strain. In many circumstances, access to the deposited strain by third parties can be made dependent upon the signing of an agreement, for example a material transfer agreement or MTA.

For strains that have not been published, the repositories often provide an embargo on third party access. This embargo may be for a defined period of time or until publication.

Researchers / Institutions depositing strains to the APB maintain ownership of the strain and may request access by third parties be dependent upon the signing of an agreement. The depositor may also waive all rights to the strain permitting unrestricted access and distribution.

The Australian Phenome Bank offers a 2-year embargo for unpublished strains.

## **9. Databases**

While each of the repositories listed above support online tools for searching for strains of interest held in their archive, each of the repositories also list their strain holdings with the International Mouse Strain Resource (IMSR).

The web address for this resource is: <http://www.findmice.org//index.jsp>.

The ES cell clones carrying targeted knockouts or conditional knockouts can be searched for and identified through the International Knockout Mouse Consortium (IKMC) webportal: <http://www.knockoutmouse.org/>

## **10. Shipping**

Genetically modified mouse strains are continually being transported from one research facility to another. While the common practice is to ship live mice there are animal welfare issues to consider. Extreme weather conditions, turbulence and continual access to nutrition can severely affect the health of the animals.

In addition, many transport companies and airlines will not transport live mice. Thus, the research community needs to become more dependent upon transportation of mouse strains as frozen stock.

The introduction of liquid nitrogen dry-shipping units has made the distribution of frozen embryos and gametes a simple and safe procedure. Dry shippers absorb liquid nitrogen into the heavily insulated walls of the unit so that the contents remain at ultra-cold temperatures for up to 2 weeks. Importantly, since dry-shippers do not contain free-flowing liquid nitrogen they are not classified as “dangerous goods” by the International Air Transport Association, minimising costs and bureaucracy associated with shipping.

The APB has several dry shippers at its disposal and vast experience with shipping frozen material to the US, Europe and Asia. International shipments require prior approval from AQIS. Exports require AQIS authorised and signed documentation and imports require an AQIS import permit. APB staff have an excellent working relationship with AQIS staff.

## **11. Importation of frozen material into Australia**

Under current regulations directed by BioSecurity Australia, frozen gametes and embryos can only be imported into Australia if the processing facility tested the gamete or embryo donor animals for Hantavirus. Unfortunately, the majority of cryopreservation facilities do not test for Hantavirus as this pathogen is considered not to be a risk to mice housed in research facilities. Thus, importation of frozen gametes and embryos into Australia is almost impossible, especially since the majority of the frozen material was archived many years ago and the donor colony long gone.

The APB has made a submission to BioSecurity Australia for the current regulations to be reviewed and the requirements for Hantavirus screening be removed. It is the understanding of the APB that this review is currently underway.

## **12. Acknowledgment**

Material for this document was sourced from work produced by Naomi Nakagata, Center for Animal Resources and Development, Kumamoto University, Japan and Martin Fray, Head of Biological Resources, MRC Harwell, United Kingdom. The APB was established with funding from the NHMRC awarded to Prof Chris Goodnow (ANU), Prof Moira O’Byrne and Prof David DeKretser (Monash University) as well as numerous associate investigators.

In addition, funds provided to the Australian Phenomics Network from the Australian Federal government through the NCRIS and EIF initiatives have been allocated to assist the running of the APB.

## 13. Appendix 1

### 13.1. Cryopreservation techniques

As a large component of the sperm or embryo is water, when the temperature drops (during the freezing process), this water is drawn out of the cell, resulting in cell shrinkage, and it starts to freeze extracellularly. Other solutes within the cell become very concentrated. Ice formation begins around the cells, causing crushing of the cell and the ice can penetrate and damage the cell membrane. The combination of which results in death of the sperm or embryo. Successful cryopreservation techniques prevent the formation of the ice crystals by the manipulation of the osmotic balance of the cell and the rate at which the cell is cooled. Long exposure to the cryoprotectant is also toxic to the cell, so the thawing procedures used are just as important as the freezing procedures. In most cases the choice of thawing procedure is determined by the freezing procedure used.

#### 13.1.1. Sperm

Slow and rapid methods can be used to cryopreserve sperm. The rapid procedure involves freezing the sperm as pellets in tubes inserted into holes in a block of dry ice. The temperature is reduced from +20°C to -80°C at a rate of 200°C to 250°C/min. The tubes are then transferred to liquid nitrogen and the temperature drops to -196°C at 7000-8000 °C/min. Samples are then transferred to liquid nitrogen cooled storage vessels.

During the slow-cooling procedures, sperm are aliquoted into vials or straws and then subjected to a slower temperature reduction of +20°C to -170°C at a rate of 20-60°C/min and then from -170°C to -196°C more slowly at approximately 2-5°C/min. These cooling rates are achieved with gas phase liquid nitrogen and the plunging into liquid nitrogen. Samples are then transferred to liquid nitrogen cooled storage vessels.

In recent times the slow-cooling method has become the most relied upon procedure. Cryopreservation of sperm isolated from C57BL/6 strains has been notoriously difficult. C57BL/6 sperm post-thaw viability and motility are usually low resulting in poor fertilisation rates. Recent advancements to the slow-freezing procedure along with significant changes to the IVF method, published by Nakagata's group, have resulted increased C57BL/6 sperm survival and dramatic improvements to the fertilisation rates. The APB uses the slow-cooling method and recently adopted changes proposed by Nakagata.

Technological advancements have made it possible to obtain live born pups from IVF conducted using freeze-dried sperm and sperm frozen in the absence of cryoprotectants. Live born pups have also been produced from sperm recovered from whole testis frozen at -20°C for 15 years. Unfortunately, the sperm preserved under these conditions is immotile and fertilisation can only be achieved using intracytoplasmic sperm injection (see below). One advantage of storing freeze-dried sperm is the sperm remains viable after long-term storage at 4°C. This eliminates the expense and inconvenience of liquid nitrogen storage and facilitates easy transportation of samples.

### 13.1.2. Embryos

It is possible to freeze all pre-implantation stages of embryos as well as oocytes. Multiple protocols using a variety of cryoprotectants exist. Methods of cryopreservation can be divided into two categories, equilibrium and non-equilibrium.

During equilibrium methods, embryos are cooled slowly (0.2-2°C/min) to -80°C. The procedure is quite time-consuming and requires specialised equipment (slow-rate freezers). Embryos are resuspended in cryoprotectant and then subjected to the cooling process. Successful cryopreservation is achieved by the gradual dehydration of the embryos as water is drawn from the intracellular compartments by extracellular ice crystallisation combined with ingress of the membrane permeable cryoprotectant.

The degree of dehydration is determined by the duration of the cooling cycle. Upon completing of the cooling phase, the embryos are plunged into liquid nitrogen.

The degree of dehydration determines how the cells should be thawed. Embryos cooled to -70 - -80°C are highly dehydrated and must be thawed slowly to achieve adequate rehydration.

In non-equilibrium cooling, embryos are briefly incubated with high concentrations of cryoprotectant (resulting in dehydration) and cooled rapidly (>200°C/min) by plunging straight into liquid nitrogen or exposing the embryos to the vapour phase of the liquid nitrogen. When the cryoprotectant concentration is higher than 40%(v/v) or 5M the supercooled solution becomes so viscous that it solidifies into a glass-like state without forming ice crystals (vitrification). The fast, relatively simple nature of the rapid procedures along with the absence of requiring expensive equipment are the greatest advantages of the non-equilibrium methods.

### **13.2. Recent advances for sperm cryopreservation**

Freezing and thawing dramatically reduce the fertility of C57BL/6 mouse sperm by reducing motility and ability to penetrate the zona pellucida (ZP) and inducing plasma membrane damage to the acrosomal region.

In July 2011, Naomi Nakagata and Toru Takeo published a manuscript describing an improved procedure for mouse sperm cryopreservation and IVF.

**Their claim is the new procedure improved IVF with sperm from C57BL/6 mice from 10% to 90% 2-cell efficiency.**

The improvements are due to:

- The addition of L-glutamine to the cryoprotectant,
- Inclusion of MBCD to the media used for post-thaw incubation of the sperm,
- The inclusion of reduced glutathione in the IVF media.

At a meeting in Madrid in 2012, a group of leading mouse IVF service providers gathered to discuss the advances announced by the Nakagata group. The European Mouse Mutant Archive (EMMA) have written an easy to follow procedure based on the finding of the Nakagata group. It was clear from this meeting these procedural advances have been adopted by many laboratories around the world, the APB included.

#### **Detail:**

##### **13.2.1. L-Glutamine**

L-glutamine is the most abundant free amino acid in the plasma and tissues and plays an important role in nitrogen metabolism and protein synthesis. Several studies have reported improved cryoprotection of sperm when amino acids are added to the cryoprotectant. The addition of glutamine enhances post-thaw motility and stabilizes the plasma membrane in mammalian sperm. However, mechanisms regarding the cryoprotective effect of L-glutamine on mouse sperm are not fully clear.

##### **13.2.2. methyl- $\beta$ -cyclodextrin (MBCD)**

Previously, the Nakagata group (in 2008 & 2010) had shown the post-thaw incubation of the sperm in media containing methyl-beta-cyclodextrin (MBCD) dramatically improved the fertility of frozen/thawed C57BL/6 mouse sperm. MBCD is a methylated cyclic heptasaccharide consisting of  $\alpha$ -(1-4)-glucopyranose units with a hydrophilic outer surface and a central lipophilic cavity. Its unique structure gives MBCD the ability to form inclusion complexes with many lipophilic agents. During sperm preincubation, MBCD promotes cholesterol efflux from the plasma membranes of sperm. Cholesterol efflux stimulates protein tyrosine phosphorylation. It is assumed that a loss of cholesterol induces a change in the stability and distribution of lipid rafts. Lipid rafts are thought to function in signal transduction at the cell surface in response to intracellular and extracellular stimuli. A change in the architecture of lipid rafts may regulate an influx of  $\text{Ca}^{2+}$  ion coupled with the release of intracellular  $\text{Ca}^{2+}$ , leading to the opening of store-operated channels (SOCs) in the plasma membrane.

### 13.2.3. **Reduced Glutathione**

The addition of reduced glutathione (GSH) to a fertilization medium was reported by Sue Bath, Melbourne, to increase the fertility of frozen/thawed sperm of various strains of mice. GSH is a biological antioxidant that protects mammalian sperm against the loss of DNA integrity and motility through oxidative stress.

Takeo and Nakagata showed the protective effect of GSH is due to the formation of free thiols. The GSH-stimulated fertilization was associated with an increase in the number of free thiols in the zona pellucida (ZP) and ZP expansion. GSH is a biological antioxidant that reduces disulfide bonds (-S-S-) to produce two thiol groups (-SH). Disulfide bonds between cysteines are vital for the formation of stable native conformations in proteins.

The mouse ZP consists primarily of three glycoproteins, namely ZP1, ZP2 and ZP3, which have 21, 20 and 12 cysteine residues, respectively, which form intra- or intermolecular disulfide bonds. It's been reported that ZP1, which forms homodimers linked by intramolecular disulfide bonds, plays a role in linking filament sheets composed of ZP2 and ZP3 to maintain the ZP structure.

Takeo and Nakagata found that high GSH concentrations increased the perivitelline space and increased the level of free thiols in the ZP. Breaking disulfide bonds in the ZP may alter protein interactions thereby modifying the ZP structure and function. Zona dissection using GSH appears to facilitate freeze / thaw sperm ZP penetration and fertilization.