May 2011

منظمة الأغذية والزراعة للأمم المتحدة | 联合国粮食及农业组织 | Food and Agriculture Organization of the United Nations | Organisation des Nations Unies pour l'alimentation et l'agriculture | Продовольственная и сельскохозяйственная организация Объединенных Наций | Organización de las Naciones Unidas para la Agricultura y la Alimentación

Item 5.2 of the Provisional Agenda

COMMISSION ON GENETIC RESOURCES FOR FOOD AND AGRICULTURE

Thirteenth Regular Session

Rome, 18 – 22 July 2011

DRAFT GUIDELINES FOR THE CRYOCONSERVATION OF ANIMAL GENETIC RESOURCES

INTRODUCTION

1. This document presents draft guidelines for cryoconservation of animal genetic resources, for the information of and endorsement by the Commission. The continual erosion of the diversity of animal genetic resources stands as a threat to long-term global food security. *Ex situ* conservation programmes involving *in vitro* storage of cryoconserved germplasm can contribute to the maintenance of this diversity and thus help to ensure that it will be available to allow livestock keepers and animal breeders to confront future changes in production and economic environments. Nevertheless, many countries have no such programmes, in part because a lack of t

2. The *Global Plan of Action for Animal Genetic Resources* highlights the need to establish or strengthen *ex situ* conservation programmes[1] and to strengthen human capacity for management of animal genetic resources, including conservation[2]. FAO took the initiative to develop technical guidelines for cryoconservation of animal genetic resources and initiated the process with a broad-based working group of experts, each of whom contribute to activities related to cryoconservation of animal genetic resources within their respective countries. The guidelines were then reviewed, tested, validated and finalized at training workshops held in Tunisia, Ecuador and the Netherlands. More than 120 scientists, technicians and decision makers attended these workshops.

3. The document *Draft guidelines for the cryoconservation of animal genetic resources*[3] was presented to the sixth session of the Intergovernmental Technical Working Group on Animal Genetic Resources (Working Group), which requested FAO to revise the guidelines in the light of comments received from members of the Working Group and to make the revised guidelines available to the Commission. FAO received comments from Canada and Germany. The revised guidelines are contained in this document.

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[1] *Global Plan of Action for Animal Genetic Resources*, Strategic Priority 9. ftp://ftp.fao.org/docrep/fao/010/a1404e/a1404e00.pdf

[2] *Global Plan of Action for Animal Genetic Resources*, Strategic Priority 14.

[3] CGRFA/WG-AnGR-6/10/Inf.8.

4. Upon endorsement by the Commission, FAO will publish the guidelines for cryoconservation of animal genetic resources and distribute them widely as part of a series of publications prepared by FAO to support countries in the implementation of the *Global Plan of Action for Animal Genetic Resources*. The guidelines are intended for use by policy-makers and gene bank managers and technicians. They will provide a decision support tool to assist interested countries to confirm the need for a national cryoconservation programme, determine its objectives and priorities, establish the necessary infrastructure and human resources and collect and safely preserve the most valuable animal genetic resources.

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FOREWORD

Livestock agriculture is in a period of tumultuous change and upheaval. General economic development and population growth and mobility in the world has increased demand for livestock products, but has also introduced pressures on the sustainability of rural environments and animal production systems. Livestock keepers will need to increase their efficiency to meet this demand while continually adapting their genetic resources to constantly changing economic and environmental climates.

The genetic diversity necessary to allow for this adaptation is in a state of continual decline. The animal genetic resources that remain are not utilized in the most efficient way. The *State of the World's Animal Genetic Resources for Food and Agriculture*[4] confirmed that a significant proportion of the world's 7000+ livestock breeds are at risk of extinction and that many countries lack the technical capacity to ensure the proper management and sustainability of their animal genetic resources.

To formally recognize and address these problems, the Member States of the FAO developed the *Global Plan of Action for Animal Genetic Resources*[5] *(Global Plan of Action)*, which was adopted at the First International Technical Conference on Animal Genetic Resources for Food and Agriculture in Interlaken, Switzerland in September 2007. The *Global Plan of Action* contains four Strategic Priorities Areas that provide a basis for enhancing sustainable use, development and conservation of animal genetic resources throughout the world. The Interlaken Conference called on FAO to continue developing technical guidelines and providing assistance and to continue coordinating training programmes as a means to support countries in their efforts to implement the *Global Plan of Action*.

Conservation of animal genetic resources is the third Strategic Priority Area. Conservation involves both the *in vivo* maintenance and management of genetic diversity within the populations of livestock that are actively contributing to the livelihood of their keepers and the nutritional health of the general population, as well as the *in vitro* storage of genetic material that can be introduced at a later time to increase or introduce diversity into the live populations. In a previous edition of guidelines, FAO had covered both of these topics in a single publication, the *Secondary Guidelines: Management of Small Populations at Risk*[6]*,* which was released in 1998. Given the technological advances and increase in information available in the past decade, conservation of animal genetic resources will now be addressed in separate publications, *Guidelines for In Vivo Conservation of Animal Genetic Resources* and this document, *Guidelines for the Cryoconservation of Animal Genetic Resources.*

The development and operation of a gene bank for cryoconservation of animal genetic resources requires technical capacity in genetics, reproductive physiology, cryobiology and data management. In addition, coordination among a wide group of stakeholders is necessary in the development of policies and procedures regarding establishment, operation and long-term sustainability of the gene bank. These guidelines were developed to provide an overview of the fundamental issues of concern in the development and operation of gene banks as a complementary piece of a comprehensive national strategy for management of animal genetic resources.

ACKNOWLEDGEMENTS

Seven international scientists were responsible for the majority of the technical content of these guidelines, Harvey Blackburn, Robert Godke and Phil Purdy (USA), Sipke-Joost Hiemstra and

[4] http://www.fao.org/docrep/010/a1250e/a1250e00.htm

[5] http://www.fao.org/docrep/010/a1404e/a1404e00.htm

[6] http://www.fao.org/ag/againfo/programmes/es/lead/toolbox/Indust/sml-popn.pdf

Henri Woelders (Netherlands), Arthur Mariante (Brazil) and Flavia Pizzi (Italy). All of these persons make substantial contributions to the management of animal genetic resources within their respective countries, including the operation of national or regional gene banks.

The guidelines were reviewed, tested, validated and finalized at training workshops held at the National Gene Bank of Tunisia, the Escuela Superior Politécnica del Litoral in Ecuador, and the Centre for National Genetic Resources of the Netherlands, which were organized with the local assistance of M'Naouer Djemali and International Centre for Agricultural Research in the Dry Areas (ICARDA), Paul Herrera Samaniego and Paul David Silva, and Mr. Hiemstra, respectively. In addition to FAO, significant financial support was provided by the United States Department of Agriculture, the European Regional Focal Point for animal genetic resources and the various host organizations. More than 120 scientists, technicians and decision makers attended these workshops.

The guidelines were prepared under the supervision of Paul Boettcher, with the full support of the Chief of FAO's Animal Genetic Resources Branch, Irene Hoffmann, and of current and former officers of the Animal Genetic Resources Group: Badi Besbes, Beate Scherf, Roswitha Baumung, and Dafydd Pilling. Administrative and secretarial support was provided by Kafia Fassi-Fihri and Silvia Ripani.

FAO would like to express its thanks to all these individuals and to those not mentioned here who generously contributed their time, energy and expertise.

THE GOAL AND STRUCTURE OF THESE GUIDELINES

The objective of these guidelines is to provide technical guidance and to serve as a decision aid among the available cryoconservation options and in the design and establishment of animal gene banks. These guidelines are written under the assumption that the reader has already decided that cryoconservation is likely to be the most logical approach for conservation of the animal genetic resources of interest. The considerations and reflections are intended to be relevant to all species of domestic livestock and where appropriate species-specific guidance is given. Much of the information may also apply to cryoconservation of wild relatives and other wildlife species of interest and countries may consider developing joint gene banks that may share some facilities for both domestic and wild animals.

These guidelines will emphasize cryoconservation of animal genetic resources. Matters related specifically to *in vivo* conservation as well as general issues of conservation are to be found in the *FAO Guidelines for In Vivo Conservation of Animal Genetic Resources*.

This document is designed to provide the necessary technical background for countries wanting to set up, implement and monitor gene banks. Although reading of all chapters is recommended, certain chapters are targeted to specific stakeholders with a specific technical interest and responsibility with respect to operating a gene bank.

Chapter 1 reviews the reasons to conserve animal genetic sources and compares the various conservation options. The comparison is designed to help the reader to confirm that cryoconservation is the most appropriate option for the animal genetic resource to be conserved, based on the assumption that a conservation programme for that resource is needed.

Chapter 2 discusses what must be done before the freezing and storage of germplasm can actually start, addressing the preparation, implementation and organization of gene banks.

Chapter 3 presents the objectives of conservation that gene banking programmes can address.

Chapter 4 describes the types of germplasm and tissue that can be cryopreserved, as well as their utilization, so that an informed choice can be made regarding the material to be stored.

Chapter 5 presents the requirements and costs to establish gene banks of different sizes and technological sophistication.

Chapter 6 deals with the genetic issues that are to be considered when designing and implementing a cryoconservation programme, particularly considering the amounts of various types of germplasm to store to capture desired amounts of genetic diversity. Biological material undergoes a number of notable and sometimes drastic changes when subject to cryopreservation and some of these changes will decrease the viability of the conserved germplasm.

Chapter 7 therefore describes the process of cryoconservation on the cellular level and its possible effects on the material subject to cryoconservation. This brief overview is designed to provide the basic information needed to diagnose and avoid damage to genetic material during the freezing process.

The available techniques for cryopreservation of animal genetic resources are often complex and, therefore, a description of collection and cryopreservation methods, according to species and the type of genetic material preserved are given Chapter 8.

Chapter 9 addresses the health and sanitary issues that must be considered when establishing and operating gene banks for animal genetic resources to help prevent the conservation of potentially dangerous pathogens along with the valuable genetic material.

Chapter 10 describes documentation and database requirements for organization of information regarding the individual animals and their samples of genetic material stored in the gene bank. To be of use, material stored in the gene bank must eventually be thawed and used to create new animals and therefore, organization and annotation of the stored material is critical to ensure its proper utilization.

Chapter 11 addresses the legal issues of cryoconservation. Although animal genetic resources can be considered a public good, the animals that will be the source of the germplasm are usually privately owned. This ownership may or may not change during the gene banking process, but the terms of agreements between gene banks and donors must be explicitly defined.

Finally, the priorities for capacity building, the need to train farmers and extension workers, as well as the inclusion of this subject in higher education are presented in Chapter 12.

These chapters are then followed by a series of Appendices that provide step-by-step instructions on procedures to be followed in the collection and cryopreservation of germplasm.

ABBREVIATIONS USED

AI:	Artificial Insemination
AnGR:	Animal Genetic Resources
AV:	Artificial Vagina
CBD:	Convention on Biological Diversity
CASA:	Computer Assisted Sperm Analysis
CIDR:	Controlled Internal Drug Release Device (Commercially marketed as Eazi Breed® in the USA)
CL:	Corpus Luteum
CPA:	CryoProctective Agent
DNA:	Deoxyribonucleic Acid
eCG:	Equine Chorionic Gonadotropin
ET:	Embryo Transfer
FAO:	Food and Agriculture Organization of the United Nations
FSH:	Follicle Stimulating Hormone

hCG: Human Chorionic Gonadotropin
IETS: International Embryo Transfer Society
IVF: *In vitro* Fertilization
IVM: *In vitro* Maturation
IVP: *In vitro* Produced
ICSI: Intracytoplasmic Sperm Injection
kg: Kilogram
L: Litre
LH: Luteinizing Hormone
LN2: Liquid Nitrogen
m^2: square meter
µg: microgram
µg: microlitre
mg: Milligram
ml: Millilitre
MTA: Material Transfer Agreement
NAGP: National Animal Germplasm Programme of the United States of America
N_e: Effective population size
NSAP: National Strategies and Action Plans for animal genetic resources
NT: Nuclear Transfer
OIE: World Organization for Animal Health (Office International des Epizooties)
OPS: Open Pulled Straw method of cryopreservation
OPU: Ovum Pick Up
PGC: Primordial Germ Cell
PBS: Phosphate-Buffered Saline
PMSG: Pregnant Mare Serum Gonadotropin
QTL: Quantitative Trait Loci
SCNT: Somatic Cell Nuclear Transfer
SOP: Standard Operating Procedure
TUGA: Transvaginal Ultrasound-Guided Oocyte Aspiration
VS: Vitrification Solution

1. CONFIRMING THE DECISION FOR CRYOCONSERVATION

Conservation of animal genetic resources (AnGR) may be undertaken for a number of reasons. In developed countries, traditions and cultural values are important driving forces, which ensure the development of conservation measures for rare breeds and promote the emergence of niche

markets for livestock products. In developing countries, however, the immediate concerns are for food security and economic development.

In a general way, the objectives for conservation of AnGR fall in the following categories: (a) economic; (b) social and cultural; (c) environmental; (d) risk reduction; and (e) research and training.

- Domestic Animal Diversity should be maintained for its economic potential in allowing response to changes in the ecosystem, in market demands and associated regulations, by changes in the availability of external inputs, by emerging disease challenges, or by a combination of these factors.
- Domestic Animal Diversity has an important social and cultural role. Loss of typical breeds, therefore, means a loss of cultural identity for the communities concerned, and the loss of part of the heritage of humanity.
- Domestic Animal Diversity is an integral part of the environment in a variety of production systems**.** The loss of this diversity would contribute to greater instability and risk, decreased ability to respond to changes of the environment. Maintenance and development of adapted breeds are of critical importance to ensure that food security can be achieved sustainably without adverse environmental impact.
- Domestic Animal Diversity should be conserved for research and training. This may include basic biological research in genetics, nutrition, reproduction, immunology and adaptation to climatic and other environmental changes.

The specific objective or objectives for conserving a given AnGR will influence the strategy employed in its conservation. Conservation strategies can be categorized as either conserving animals *in situ*, within the environment or production systems in which they were developed, or *ex situ*, in all other cases. The latter can be further divided into *ex situ - in vivo* conservation and cryoconservation.

1.1. *In situ* conservation

In the context of Domestic Animal Diversity, *in situ* conservation is primarily the active breeding of animal populations for food and agricultural production such that diversity is optimally utilized in the short term and maintained for the longer term. Operations pertaining to *in situ* conservation include performance recording schemes, development of breeding programmes, and management of genetic diversity within populations. *In situ* conservation also includes ecosystem management and use for the sustainable production of food and agriculture. Aspects of *in situ* conservation are discussed in detail in the *FAO Guidelines on Breeding Strategies for Sustainable Management of Animal Genetic Resources*[7] and *Guidelines for In Vivo Conservation of Animal Genetic Resources*.

1.2. *Ex situ* conservation

In the context of domestic animal diversity, *ex situ* conservation means conservation away from the habitat and production systems that developed the resource. This includes both storage as live animals away from the habitat and cryoconservation.

1.2.1. Ex situ - in vivo *conservation*

Ex situ - in vivo conservation is simply *ex situ* conservation, with storage of germplasm as live animals. As for *in situ* conservation, it is accepted that improvement and natural selection outside the original environment may alter gene frequencies in the gene pool. A key question for this strategy is whether or not long-term finances and commitment is available to maintain generations of animals to the standards (i.e. sufficiently large population size) required for

[7] http://www.fao.org/docrep/012/i1103e/i1103e.pdf

successful conservation. More detail on *ex situ – in vivo* conservation can be found in the *FAO Guidelines for In Vivo Conservation of Animal Genetic Resources*.

1.2.2. Cryoconservation

Cryoconservation is the collection and deep-freezing of semen, ova, embryos or tissues which may be used for future breeding or regenerating animals. A key question for cryoconservation is whether, in the short term, the facilities and expertise required for the collection of the samples can be financed and put in place. The logistics and costs of providing and maintaining storage facilities will need to be addressed before the cryoconservation is carried out.

1.3. Complementary roles of in situ and ex situ conservation

The Convention on Biological Diversity[8] emphasizes the importance of *in situ* conservation and considers *ex situ* conservation as an essential complementary activity to *in situ*[9]. *In situ* and *ex situ* conservation are complementary, not mutually exclusive. The exact strategy will clearly depend on the conservation objectives. *In situ* and *ex situ* strategies differ in their capacity to achieve the different conservation objectives.

In situ conservation is often regarded as the preferred method from a general point of view because it ensures that a breed is maintained in a dynamic state. This may be true when the dynamics of a breed are characterized by slow and balanced adaptation to conditions. However, commercially important breeds are often subject to high selection pressure and larger than desired levels of inbreeding (a few top sires fathering many offspring), whereas commercially less-important breeds often have a small population number and are threatened by genetic drift and extinction. Conserving genetic diversity by keeping live animals outside their production or natural environment (*ex situ - in vivo*) will not always be able to guarantee the maintenance of the genetic diversity of a breed. Therefore, *in vivo* conservation should be complemented by cryopreservation of germplasm. In other words, long term *in situ* conservation programs may benefit from a germplasm repository.

1.4. When is cryoconservation the best option?

As a result preparation of National Strategies and Action Plans and National Conservation Plans for AnGR, countries should have identified the AnGR that should be conserved and the respective objectives for their conservation. Table 1.1 compares cryoconservation, *ex situ – in vivo* conservation and *in situ* conservation, indicating the preferable techniques with respect to a number of conservation goals. Based on the objectives for conservation, the existing national technical capacity and infrastructure for cryoconservation, and amount of capital to invest in developing and maintaining a gene bank for AnGR, each country should determine for which AnGR, if any, cryoconservation programmes should be undertaken.

[8] http://www.cbd.int/convention/articles/?a=cbd-08.

[9] http://www.cbd.int/convention/articles/?a=cbd-09.

Table 1.1. Conservation techniques and objectives, adapted from Gandini and Oldenbroek (2007).

Objective	Technique		
	Cryoconservation	***Ex situ – in vivo***	***In situ***
Flexibility of the genetic system, as			
• Insurance for changes in production conditions	++	+	++
• Safeguard against diseases, disasters, etc.	++	-	-
• Opportunities for research	++	++	++
Genetic factors			
• Continued breed evolution / genetic adaptation	-	+	++
• Increase knowledge of breed characteristics	+	++	+++
• Minimize exposure to genetic	++	-	+
Sustainable utilization of rural areas			
• Opportunities for rural development	-	+	+++
• Maintenance of agro-ecosystem diversity	-	-	++
• Conservation of rural cultural diversity	-	+	++

Reference

Gandini. G. & Oldenbroek, K. 2007. Strategies for moving from conservation to utilisation. Chapter 2 in *Utilisation and conservation of farm animal genetic resources.* Edited by K. Oldenbroek. Wageningen Academic Publishers, the Netherlands.

2. IMPLEMENTATION AND ORGANIZATION

Once the decision to establish a cryoconservation programme is made, preparation and planning can begin. A well planned and maintained programme for cryopreservation of germplasm can play a key part in the maintenance of genetic variability within a given livestock population and virtually prevent its complete extinction. Nevertheless, a gene bank should in most cases be considered a type of insurance against loss of genetic variability or extinction and a thus complement to, rather than a substitute for effectively-designed and implemented programmes for comprehensive management of AnGR. In addition, although a facility for cryopreservation of germplasm can be relatively quickly and inexpensively established to effectively "save" breeds at a great risk for extinction, long-term maintenance of a gene bank requires continual resources and, therefore, plans to ensure the continual provision of such resources.

2.1. Shaping national strategic and action plans

Conservation is only one of the four Strategic Priority Areas in the *Global Plan of Action* and cryoconservation is only one of the several available options for AnGR conservation. For the most effective and efficient management of a country's AnGR, creation of gene banks should ideally be done after the establishment and under the framework of a national programme for management of AnGR. The FAO has developed *Guidelines for the Preparation of National Strategies and Action Plans for Animal Genetic Resources for Food and Agriculture.* These guidelines call for the establishment of a National Advisory Committee on AnGR. This

committee should either take the responsibility for planning of the gene bank or, alternatively, create a dedicated task force of experts to deal specifically with gene banking.

Many countries have existing gene banks and yet no formal National Strategies and Action Plans for AnGR (NSAP). These gene banks should be accounted for in the development of the NSAP. Of course, from a practical point of view, countries should not allow at-risk AnGR to be lost while waiting for establishment of NSAP if such loss can be prevented by creating a gene bank.

2.2. Organization and institutions

No single particular system of organization and institutions will be ideal for all situations and countries. The optimal system will depend on a wide variety of factors, including the types of existing infrastructure and related institutions, technical capacity of personnel, species of interest, stakeholders, and level of government versus private support. For example, a breed association will have a vested interest in establishing a gene bank for conservation of its breed, whereas governments may assign the greatest priority to conserving the populations that are most critical for national food security. Evaluation of the major institutions and stakeholders, their goals and their capacity to contribute to conservation programmes will be an essential step in the initial phases of the development of the NSAP.

Establishment of linkages among institutions will be critical to maximize efficiency. Collaboration with artificial insemination (AI) centres will be highly beneficial in many developing countries, as these centres will have both the technical capacity and the infrastructure for collection, freezing and storage of germplasm, as well as a reliable source of liquid nitrogen (LN2). In other situations, the collaboration may be across species and ministries. For example, efficiencies may be achieved by having a national gene bank for all organisms, including not only livestock, but also wild animals and or plants (assuming health and sanitary regulations are accounted for).

When the characteristics of a country predicate that multiple gene banks for livestock exist, linkages for communication among the gene banks and with the National Focal Point for AnGR will help the National Focal Point to determine that the conservation goals established by the NSAP are being met without duplication of activities. Although these guidelines may refer to a single national gene bank, one practical option that may be considered is to operate a “virtual” gene bank, where different types of germplasm (e.g. varying according to species or breed) may be held in different locations, but a central database is used to monitor inventories.

2.3. Participation of stakeholders

2.3.1. The State

The overall responsibility and control for conservation of AnGR within the government will usually be with the Ministry of Agriculture. Where the responsibility for biological diversity as a whole is vested in other ministerial portfolios, such as the Ministry of Environment and Natural Resources, then close liaison and co-ordination is necessary to realize effective policy, planning and operations. These relationships must all be clarified in the development of NSAP. The government as a whole ultimately influences cryoconservation programmes through budget allocation, whether this be by directly supporting state-owned and operated gene banks or by providing grants to private institutions that operate gene banks for the public interest. In many developing countries, AI centres are financially supported by the Ministry of Agriculture. Universities and research institutes as public institutions could be entrusted with the cryoconservation of endangered breeds, possibly to be used in part for research purposes.

As previously indicated, other important responsibilities for the State include overseeing the development of NSAP, establishing a National Advisory Committee on AnGR or cryoconservation task force, co-ordinating national activities involving all stakeholders, providing funding and training, promoting linkages, and providing basic building blocks for regional and international collaboration.

2.3.2. Individual farmers and breed associations

The private farmers will typically be the initial owners of the individual animals whose germplasm is to be cryopreserved. Thus, their engagement will be critical for the success of the gene bank. The individual farmers may provide information about origins of breeds and animals, to assist in the process of selecting genetically unrelated stock as much as possible. Formal agreements must be drawn up to outline terms of any compensation for provision of germplasm to the gene bank and rights to and conditions surrounding future access to the stored material (see Chapter 12).

Breed associations such as co-operative breeding and herd-book associations may see it as their responsibility to maintain breeds. As an organization they are clearly interested in the long-term well-being of the breeds and may organize and financially support cryoconservation activities. In any case, support from breeders' organizations is necessary for good survey information for selection of animals whose germplasm is deposited the gene bank and for the general success of conservation schemes. Other Non-governmental Organizations (NGO), in addition to breed associations, may also be able to contribute to cryoconservation programmes, in particular through grass-roots interaction with farmers and breeders. Some NGO have breed conservation as their specific objective.

2.3.3. Private companies

Commercial breeding companies, processing companies and agricultural support services may become more interested and increasingly involved in cryoconservation activities (particularly pig and poultry businesses) in order to maintain the variation of breeds and the possibility to access these breeds easily when producing new founder lines. Private companies continue to seek additional genetic resources outside the company, and are likely to conserve genetic material that may hold future promise, and are responsible for research that directly benefits them. They may have the infrastructure available to host a public gene bank. Clearly, under such a scenario, precise legal agreements regarding access and benefit sharing will be critical to ensure total transparency (see Chapter 11).

2.3.4. The National Coordinator for AnGR

The FAO National Coordinator (NC) and National Focal Point for AnGR will be important partners and likely a member of the National Advisory Committee on AnGR. The NC should be kept informed about all activities on cryoconservation. The NC will have responsibility for reporting this information to the FAO.

2.4. Funding and attracting support for projects

As mentioned previously, the immediate stakeholders such as the State, breeders associations and private companies will generally be expected to provide most of the financial support for the gene bank. However, other sources of funding may be necessary. In order to develop plans that may attract funding and wider support, there generally must be clear relevance to the Convention on Biological Diversity, which implies relationships of the domestic livestock to: (i) conservation of biological diversity; (ii) sustainable use; and, (iii) equitable sharing of benefits from use. The priorities for funding of a project by national governments and co-operating international bodies will increase if it can be demonstrated to be of relevance to multiple aspects of government policy, such as agricultural, environmental, cultural, social, and where draught animals are involved, energy and transport.

Documenting the wider importance of a local breed raises it from being more than a commodity, subject to market-driven economic forces, and allows it to be valued according to the principles of the Convention on Biological Diversity. The participants in the projects, ranging from those that provide finance to those that contribute in-kind through services rendered, may then extend beyond those international and national agencies concerned with agriculture and domestic livestock, to those concerned with environmental issues and indigenous cultures. Increased

awareness among the general public - who are increasingly urban in their lifestyle - of problems that concern the rural community can also play an important role in influencing decisions on funding.

With respect to international agencies, there are two key features that animal conservation projects should aim to provide in order to attract funding:

- The project needs to be part of a national strategy for conservation of the whole environment taking the entire ecosystem into account, including wildlife, plants and forests, water, soil and microbes, inasmuch as animals cannot be viewed in isolation from their environment.
- The project supports indigenous communities who wish to continue conventional lifestyles. The needs of indigenous people have growing international recognition because it is now acknowledged that indigenous people have been practising sustainable lifestyles for millennia. Hence, projects targeted at encouraging use and conservation of traditional breeds with intimate ties to these communities are more likely to be viewed favourably by aid agencies.

It is difficult to get long-term funding from international aid agencies. Therefore, there should be financial commitment by governments to continue the conservation projects, and the projects should also develop conservation plans for breeds at risk in conjunction with their continued use.

3. OBJECTIVES OF CRYOPRESERVATION PROGRAMS

3.1. Gene banking

Cryopreservation allows virtually indefinite storage of biological material without deterioration over a time scale of at least several thousands of years (Mazur, 1985), but probably much longer. This means that we can preserve the present wealth of genetic diversity in long-time storage in a biological “safe deposit vault”.

Gene banks and the collections of germplasm and tissue they curate can be multifaceted in their function and objectives. While the primary gene bank function is the conservation of AnGR for use in the near, medium or long term, additional uses of the material collected exist. For example, such resources can be used to introduce genetic diversity into *in vivo* populations and thereby reduce inbreeding levels and broaden breed diversity in the event of a bottleneck or to provide flexibility to the industry when particular selection strategies are found to be, after the fact, not as appropriate as initially envisioned.

Developing gene bank collections with multiple functions is beneficial because it increases the potential returns for developing germplasm/tissue collections. Clearly, as gene bank managers proceed in developing such collections, the multi-functional role of the gene bank has to be considered in planning and executing collection strategies (ERFP, 2003).

One common purpose of a germplasm repository is to provide the possibility of recreating breeds or breeding lines in case they are lost as a consequence of a calamity. Storage of germplasm for this purpose would typically be long-term storage, without frequent use of the stored material and without the need of regular updating of the collection.

A second way to make use of gene bank resources is to support *in vivo* conservation. Frozen semen and embryos can be used to minimise inbreeding and genetic drift in small managed populations, and the combination of live animals and cryopreserved germplasm can be a powerful tool in their conservation (Meuwissen, 1999). Sonesson *et al.,* (2002) proposed a scheme where semen is collected from the first two generations and used alternatively on females, allowing a reduction of the rate of inbreeding.

Additionally, gene bank resources may be used as a back-up in case genetic problems occur. Decrease of effective population size (N_e – See Box 3.1) and the resulting high level of

inbreeding can lead to an increased frequency of specific deleterious alleles that were not apparent in a larger population. Although most individuals are likely to carry some deleterious alleles, their effects are usually recessive and only observed in the homozygous state. Small N_e increases the probability that mates will share a common ancestor and thus carry the same deleterious allele(s), which may then be passed by each parent to their offspring. This happens not only in rare breeds but can also be found in large commercial breeds (e.g. when a very small number of sires are responsible for a very large number of offspring). In such cases, the N_e of the gene pool is still very small, even if real population numbers are large. Gene bank resources may be needed to dilute the deleterious alleles in the population by introducing new genotypes (e.g. semen doses) from the original (larger) population (i.e. before selection).

Box 3.1. Maintenance of Genetic Diversity

The primary driver for developing cryopreserved germplasm collections is the ability to maintain and enhance genetic diversity of *in situ* populations. One common measure of genetic diversity is the effective population size (N_e), which is usually smaller than the absolute population size.

The Ne is "the number of breeding individuals in an idealized population that would show the same amount of dispersion of allele frequencies under random genetic drift or the same amount of inbreeding as the population under consideration" (Wright, 1931). An idealized population is one in which all members have an equal opportunity to produce offspring. Sex ratio has such an influence on N_e that a population composed by 4 males and 4 females has a $N_e = 8$ which is the same as the N_e presented by a population composed of 2 males and 100 females.

An N_e of 50 or larger has been recommended for rare breed conservation (FAO, 1998). At this level, rate of inbreeding is 1% per generation. However, for gene banks to reach their full service potential, additional genetic considerations are needed. For example, having sampled enough animals to capture rare alleles within a population and ensuring a breed's collection represents the range of phenotypes so the collection can be used to for corrective mating or introducing genotypes that will be useful in transitioning breeds to new market demands.

When reconstituting a breed from germplasm collections, significant attention must be given to the mating plan so that after backcrossing has been completed the genetic relationships are minimized and thereby maintaining the constant N_e. A simple approach is to use approximately equal numbers of doses of semen from each bull. The N_e can be enhanced by avoiding the use semen from a portion of males in the gene bank during the reconstitution process. These males will then be unrelated to the reconstituted animals whereby their samples can be used with the intention of increasing the N_e further once the reconstitution process is completed.

A fourth important use of the cryopreserved material is to allow development of new lines or breeds, or to quickly modify or reorient the evolution or selection of the population. For instance, storage of original or extreme genotypes can be of use to quickly redirect the genetic trend of a selected population. Verrier *et al.,* (2003) suggested the storage of genotypes that have extreme breeding values for specific traits, that carry rare alleles, or that represent particular founders or pedigree lines. A specific example can be the storage of material from traditionally dual-purpose cattle, which were later reoriented to beef or milk production.

Finally, gene banks can serve as the primary source of material for national scientists performing DNA research. Storage of DNA samples along with germplasm can speed the acquisition of samples for researchers and provide access to common sets of animals for genotyping research. Furthermore, gene banks can also supply multi-generational samples which can be of great utility in such studies.

Due to the broad array of functions for the gene bank's collection, it may be useful to subdivide the germplasm/tissue for each breed into various categories.

3.2. Collection goals

The collection goals for each of the above gene banking functions are dependent upon the potential use and difficulty in acquisition. Collection goals exist at the animal and breed level. It is important to be flexible in meeting both these criteria. The standard recommendation is that a conserved population (included a newly reconstituted breed) should have N_e of 50 individuals, so that the rate of inbreeding can be held at 1% per generation (FAO, 1998). However, Meuwissen and Woolliams (1994) showed that the recommended N_e to conserve genetic diversity could vary from 31 to 250 animals, depending on the mating system and other factors.

When aiming to conserve specific alleles, Smith (1984) points out that by preserving semen from 50 unrelated males a repository has a 63% chance of capturing alleles with a frequency of 0.01[10]. This latter estimate may give some gene bank managers cause for concern if they are attempting to capture unique and potentially unidentified alleles. By increasing the number of unrelated males to 100, the probability of capturing a rare allele at the 0.01 level increases to 87%. To increase the number of males in the collection requires the gene bank manager to weigh the trade-offs between the costs involved with additional collections (both acquisition and storage), the protection afforded, and the broader goals of the repository. In addition, with some breeds the population may be smaller than the targets mentioned above, in which case collections may include a large proportion of the existing male population, if not the entire male population.

Experience at some gene banks has shown that for some breeds acquiring targeted male numbers is relatively easy. This is mainly because certain breeds are available for collection at AI centres or are collected on farm. However, for some rare breeds that are widely dispersed and limited in numbers, the potential for multiple collections is limited. In such cases, acquiring samples from the recommended number of animals will be difficult in a short period and will have to be considered a long-term goal.

Minimum collection goals should be established. As will be described later in this chapter, the gene bank material may be categorized into separate “collections”, the most critical of which is the Core Collection, which contains germplasm necessary for reconstituting a breed. To establish minimum targets, gene bank managers need to determine how the germplasm will be used, with potential reconstitution being the most important. Different mating strategies exist across species and as a function of whether semen or embryos are used in the reconstitution process. Furthermore, collection goals are heavily influenced by the reproductive efficiencies that can be achieved in the process of reconstitution. This aspect of the process is critical because it directly impacts collection targets. As the reproductive efficiency increases, targeted germplasm goals can be decreased.

3.2.1. Breed reconstitution

According to Smith (1984) the number of parents sampled and amount of material (e.g. sperm doses, embryos, etc.) stored will depend on the eventual usage of the stock. If the stock is to be used as a purebred, or as a maternal breed in a crossbreeding program, inbreeding (leading to inbreeding depression) and loss of genetic variation (leading to lower responses to subsequent selection) should be avoided. A maximum level of inbreeding incurred by the storage process might be set at about 2%, equivalent to about 4 generations of inbreeding for many breeds of livestock in practice. The 2% would also be the percentage loss in genetic variation in forming the store, due to limited numbers. It would be equivalent to an N_e in storage of 25, and would be met by 25 unrelated sires with frozen semen, or by 25 parental pairs with frozen embryos. Thus, moderate numbers are likely to be adequate, though these might be increased in practice for a margin of safety. The number of frozen embryos or semen doses to store from each mating or each sire depends on the reproductive success with the frozen material and on the amount of testing, multiplication and additional uses to be made of the conserved stocks.

[10] Prob of capture = $(1-p)^{2(N)}$, where p is the frequency of the desired allele and N is the number of males with germplasm in the repository.

Smith (1984) was the first to suggest a minimum number of 25 donors, and ever since many manuals, including the FAO Guidelines for Development of National Farm Animal Genetic Resources Management Plans: Management of Small Populations at Risk (FAO, 1998) and more recently the European Guidelines for the Constitution of National Cryopreservation Programme for Farm Animals (ERFP, 2003) agree with this number.

In order to determine the number of donor animals to enter a cryoconservation program, FAO (1998) assumes that every animal is valuable and has a utility, in terms of the amount of genetic diversity it provides to the conserved gene pool. Each additional animal adds a marginally smaller amount of genetic variability, however. Therefore, one can expect to eventually reach a certain threshold above which the benefit of the additional variability saved is less than the costs to sample and conserve it. This threshold was established as 25 unrelated males for semen collection and 25 unrelated males and 25 unrelated females for somatic cells or parents of embryos (FAO, 1998). If fewer than this number is available then the animals are selected irrespective of the relationships among them. Sampling from more than 25 animals is, of course, beneficial if resources permit, although in some cases more animals do not necessarily yield more genetic variation (i.e. if many of them are closely related). To obtain DNA, 40 individuals should be sampled (as recommended in *FAO Guidelines for the Molecular Genetic Characterization of Animal Genetic resources*). The same males can be used for both semen and embryo (i.e. as sires of embryos) collection. The same individuals can be used for embryo collection, somatic cells and DNA. For DNA, it is recommended that if there are fewer than 25 individuals of one of the sexes available, then extra individuals from the other sex should be sampled to make the total number of DNA samples stored up to 50.

The ERFP (2003) also suggests to collect semen from a minimum of 25 donors. From a practical standpoint, achieving these recommended numbers of germplasm donors may be difficult, if not impossible. Logistics, existing population size and financial reasons might necessitate that fewer than 25 animals are sampled, but this will decrease the amount of genetic variation stored. In such cases, it is recommended to simply collect as much germplasm as possible. When many breeds must be conserved on a limited budget, collection of germplasm from fewer than 25 animals per breed from all breeds may be preferable to collection from 25 animals from each of a proportion of the breeds. Another practical approach to consider in the situation of limited resources (in terms of either available animals or finances) is to set 25 donors as a longer term goal, to be achieved over the span of several years. Chapter 5 addresses the numbers of doses (straws of semen or embryos) that will be needed for various conservation goals. More than 25 donors may be necessary when the number of doses produced per donor is not sufficient to obtain the required total number of doses.

In summary, when building Three principles in conservation might be proposed (Smith, 1984): (i) to conserve germplasm from many donor animals in small numbers rather than a few donors with large numbers; (ii) to choose donors that are as genetically and phenotypically diverse as possible; and (iii) to store the breeds as pure lines rather than gene pools so as to allow use of the unique combinations of traits and flexibility in combination of stocks. Another important consideration is to duplicate the amount of material and to store each one of the two sets of samples at different storing sites in order to reduce risks of loss (See Chapter 5).

3.2.2. Supporting in vivo conservation, within breed selection, and introducing variation into existing populations

Gene banks have the potential to bolster *in vivo* conservation efforts. Their primary role is to serve as the ultimate backup for *in situ* populations in the event of worst-case scenarios where an entire breed is lost (i.e., in the event of civil conflict or widespread drought).

There are less extreme circumstances where it may be desirable to utilize gene banks, such as in the event that a breed or population may benefit by the introduction of genetic variation. As noted previously, the stored material may be useful for elimination of deleterious genes or accessing

genes and gene combinations that have become valuable due to a change in selection goals. This particular function has several aspects.

- Minor or rare breeds have the potential threat of reduced genetic variation and high inbreeding levels, which may result in a loss of fertility and general vigour. Introduction of variation from gene banks can potentially alleviate this condition. However, using the gene bank for this purpose is predicated on the assumption that the gene bank contains samples of animals that have a lower than average genetic relationship with the population, which is possible to achieve through proper selection of donors that are as unrelated as possible.
- Some livestock breeding populations have been selected for one specific trait at the exclusion of others and as a result, lack the genetic variation required to effectively alter phenotypes to adjust to new market conditions (e.g., changes in the value of fat in milk). Reintroduction of genotypes present before selection started can help overcome this problem and, therefore, insuring that collections contain as much genetic variability as possible should be an important objective.
- With the advent and use of various DNA technologies, gene banks have the ability to store samples from animals of known genotypes for traits of interest. Having genotypic information will facilitate the use of animals contained in the collection.
- Storage and use of samples containing rare alleles can also support *in vivo* populations directly or indirectly through research activities.

3.2.3. Capturing specific alleles

Gene banks have an opportunity to support the livestock industry by assisting in the development of new breeds and/or incorporating alleles of interest into various *in vivo* populations. By having collected the breadth of genetic variation for specific breeds, gene bank managers can work with state or private breeders in developing new breeds that better fit the current or near-term market trends. The oldest material in the bank may be particularly helpful in such a project, as these samples will have become dated with respect to contemporary selection goals and may represent a unique set of animals and genes.

As more information is garnered through ongoing genomics research on livestock, linking DNA variation to phenotypes will increase the opportunities to scan the gene bank material for alleles of interest, which in turn can be used to form new breeds or to incorporate new alleles into existing breeds (Womack, 2005).

A relatively small amount germplasm may be required for utilizing a specific allele. Depending on the species and fertility and survival rates, as few as 20 to 30 doses of semen can have a rapid impact on the target population.

3.3. Collection categories

As gene banks for livestock species and the acquisition of germplasm are initiated, the need for different collection categories becomes apparent. For example, the French, Dutch and U.S. gene banks established different collection categories to meet their projected needs (Danchin-Burge and Hiemstra, 2003; Blackburn, 2004; 2009).

The purpose and size of the categories can vary depending upon the need of the country and their respective livestock industries. By developing such categories, the gene bank manager can better establish collection goals and know how well they are being met. Based upon the experiences in developing germplasm collections, the categories presented below are offered as examples. Gene bank managers may wish to consider using these or similar types of categories to assist in management of the banked material.

3.3.1. Core Collections

The term Core Collection has multiple definitions in the conservation community. Our use of the term will be germplasm collected and stored for potential use in a critical situation for a breed or population (e.g. reconstitution of an extinct breed, introduction of genetic variability in a living population to resolve a genetic crisis such as an extreme population bottleneck, or elimination of mutation that poses a threat to the population). The Core Collection is not necessarily static, but rather it is updated as needed to insure the genetics contained in it are of utility to the livestock keepers raising the breed in question. Due to variability in viability, pregnancy and survival rates, it is suggested that Core Collections are at least 150% of what is expected to be needed to reconstitute the breed.

3.3.2. Historic Collection

As genetic change occurs in the *in situ* population, the Core Collection will need to be revitalized. As a result, gene bank managers have to decide whether or not to form a Historic Collection of germplasm or to de-access the material (implying the destruction of the germplasm). This material still has value if selection goals change drastically and can be used for DNA research as well as for research projects looking for genes or gene functions, and studies on genetic diversity.

3.3.3. Working Collection

This Working Collection is the most dynamic subset of the gene bank and serves the role of providing ready access to relevant germplasm for a) creation of research populations of animals, b) introduction of unique germplasm if a breeding organization should need to mildly modify selection goals, or c) the development of a new breed.

3.3.4. Evaluation Collection

The gene bank manager must quantify the success the of the cryopreservation process for each animal sampled for the gene bank, particularly for semen. The Evaluation Collection allows for that type of activity, which should be done soon after freezing and repeated if there is any concern that the samples were compromised in some fashion. For each animal a relatively small portion of the cryopreserved germplasm is used for this purpose (e.g., 2 to 10 straws for semen). Samples from this collection may also be used to test for evidence of disease pathogens in the material. Deep-frozen germplasm is assumed to be practically 100% resistant to deterioration, at least within the reasonable time-horizons required for AnGR banking. Various studies have demonstrated that storage time has no detectable effect on viability of cryopreserved germplasm. (See Box 3.2). Nevertheless, for prudence, it is recommended to periodically thaw and check a small portion of the collection for viability at least once every 10 years.

Box 3.2. Viability of germplasm after long-term storage

It is assumed that once good quality germplasm is frozen in liquid nitrogen it should remain viable indefinitely; however, its viability has not been systematically evaluated after being stored for long periods of time.

In order to study the viability of aged frozen semen, Carwell *et al.,* (2009) inseminated 40 purebred lactating Angus cows and heifers and 88 lactating crossbred beef cows with frozen-thawed semen from 25 purebred Angus bulls, processed during three time periods (1960-1975; 1976-1991; and 1992-2002). The authors showed that overall pregnancy rates did not differ across time periods for both Angus and crossbred cows, and concluded that the semen collected from the 1960s through to 2002 is still viable, producing similar pregnancy rates in artificially inseminated beef females.

After transferring 414 sheep embryos stored for 13 years, Fogarty *et al.,* (2000) concluded that embryos cryopreserved for a considerable number of years can be successfully thawed and transferred to recipient ewes, to reconstitute a sheep population.

3.4. Utilization of the Working Collection

All of the above uses for AnGR stored in a gene bank require the development of a component of the stored material that has previously been termed the Working Collection. Germplasm stored in the Working Collection can be much more freely accessed in order to support the needs detailed above.

The Working Collection has a wide range of uses; to construct and maintain such a collection several considerations include:

1. Establishing rules for transferring to the Core Collection the germplasm that is no longer actively needed.
2. Determining when quantities of germplasm from a given donor exceed requirements for the Core Collection and can be moved to the Working Collection.
3. Locating and obtaining samples from animals of interest to deposit in the Working Collection, such as animals that may have unique gene combinations relative to the live breeding population.

The Working Collection will usually comprise primarily semen. In some countries it may be advantageous to establish linkages with AI centres and obtain germplasm samples from animals currently being collected and/or to agree to accept samples that no longer have commercial value.

Because of its broad array of uses, the Working Collection has the potential to be larger than the Core Collection for a specific breed. Furthermore, gene bank collections are not static, as germplasm needs to be continually added and de-accessed. The Working Collection is usually the most variable element of a breed's germplasm collection. Therefore, a major consideration in determining the size of the Working Collection is the available physical storage space in the gene bank, the number of cryotanks the facility can accommodate, and the recurrent financial requirements.

Given these considerations, a reasonable expectation is that Working Collections for single breeds will range in size from 50 to 200 animals and from 500 to 1,000 insemination doses.

3.5. Refreshing the collections

A common but largely misdirected criticism of gene banks is that the germplasm may lose industry relevance over time as *in vivo* populations change. This criticism assumes that once a collection is developed, new acquisitions are not made. However, as observed with plant gene banks, collections are continually expanded with new varieties. A similar approach is envisioned for livestock and would also include acquiring samples from single animals where seemingly new or otherwise interesting mutations may be evident. In addition to adding new germplasm samples to the repository, there may also be a need for gene banks to de-access material over time and as more information is garnered about the uniqueness and utility of samples in the collection. De-accession may also be necessary due to financial or physical constraints. De-accession is a difficult undertaking and a gene bank needs a well established protocol before initiating this process. Potential reasons for de-accessing samples include:

1. Genetics of the sample are too closely related to other samples in the collection (e.g. half-sibs or closer).
2. Sample post-thaw quality is low and similar genetics are in the repository.
3. Samples are sufficiently dated and judged not to be of value compared to more current samples that are being collected.

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4. POTENTIAL USE OF GERMPLASM AND TISSUE TYPES

For the conservation of genetic diversity, storage of semen, embryos, oocytes, and somatic cells, among others, have been considered. There are clear differences in present day feasibility and practicality of these possibilities.

> **Semen** has been successfully frozen and is now being widely used with AI. Sperm sexing technology has been developed for farm animals and is presently being introduced in the commercial market in some countries. Banking of sexed semen could potentially decrease the number of doses required for storage, depending on the goals of the gene bank, but increases the cost per dose substantially.

Embryos are also widely used for gene banking, but up to now their functionality has been limited to a smaller number of farm animal species relative to semen. Embryos have an advantage over semen in breed reconstitution in that they allow the recovery of the entire genome (i.e. no backcrossing is required) and thus the reconstitution can be accomplished in a single generation.

Oocytes are the female germ cell or gametocyte and may be considered for storage in gene banks. Ideally, oocytes will be frozen along with semen of the breed to be conserved, as otherwise backcrossing with semen from another breed will be necessary to reconstitute an extinct population. Relative to semen and embryos, techniques for freezing and thawing of oocytes still require more development and refinement, as well as field evaluation. An advantage of oocytes over embryos is that through *in vitro* fertilization (IVF) desired matings can be selected at the time of thawing, rather than at the time of freezing. Cryopreservation of ovaries could be another way to preserve AnGR, either as a source of oocytes or as a source of ovarian tissue for transplantation (See Section 4.7.1).

Somatic cells could be a prudent back-up where cryoconservation of gametes and embryos is not financially or technically feasible or have very low success rates. In its most simple application, the banking of somatic cells requires only the collection and direct freezing of a piece of tissue, such as a section taken from the ear. Since the creation of "Dolly", the sheep, the first animal produced by cloning of somatic cells (Wilmut *et al.,* 1997), this technology has produced live offspring of many domestic species, including cattle, goats, swine and horses. If production of live animals from somatic cells is developed to where it becomes both reliable and economical, then preservation of somatic cells would become an attractive option for cryoconservation of AnGR.

Nuclear DNA storage has been proposed for gene transfer, but these techniques still pose some difficulties. Presently, DNA is not used for re-establishing live animals but can be useful for characterization to support animal conservation decisions, including evaluation of the genetic structure within and between populations and identifying genes with effects on productivity and adaptation. See the *FAO Guidelines for the Molecular Genetic Characterization of Animal Genetic resources* for more information. DNA analysis can also improve management of gene bank inventories.

Other types of material can be stored in the cryobank for animal evaluation purposes, such as blood and serum (e.g. for future veterinary diagnostic screening and evaluation).

4.1. Semen

A major advantage of semen for cryoconservation is that existing technologies allow it to be collected and used in many animal species. Moreover, for a number of species, notably in cattle, small ruminants, horses and pigs, AI centres with dedicated animal housing and semen collection and processing facilities exist in many countries, which could facilitate the acquisition, storage and future use of semen. Field collection may be an option to consider if such dedicated facilities are not available and may be the only option for collecting material in areas where extensive farming is the norm.

Relative to other types of germplasm, semen has the disadvantage that only a single complement of chromosomes is preserved. If only semen is conserved and the cryoconserved breed was completely lost, then another breed of the same species would be needed to provide the founder females for insemination (or the female gamete in the case of other reproductive techniques). A series of backcrossings would then be required to restore the breed to its original (nearly) genetic status. By using only semen from the breed that must be recovered in each generation, the percentage of genes from the founder breed decreases logarithmically, while the percentage of genes of the breed to be recovered increases. This means that enough semen must be available to inseminate the required number of animals in the series of consecutive crosses. For recovery of a lost breed it will take at least 4 to 5 generations of backcrossing to restore the "original" genotype,

depending on the desired purity of the restored breed (Ollivier and Renard, 1995) (see 3.2.1. Breed reconstitution). Marker-assisted breeding may be used to help speed this process to a certain extent. DNA markers can be used to select progeny that contain the greatest percentage of the recipient strain genome at each generation, a process known as "speed congenics" (Hospital *et al.,* 1992; Wakeland *et al.,* 1997).

In addition to the need for backcrossing, the use of semen has the disadvantage that mitochondrial genes are not preserved; whereas, variation in mitochondrial genes between breeds and within breeds has been established (Loftus *et al.,* 1994; Troy *et al.,* 2001). In avian species, the use of semen to restore a breed would result in the complete loss of the W chromosome from that breed, as the male is the homogametic sex and has no W chromosome.

In some cases, semen collection through standard procedures can be problematic, for instance when the animals cannot be trained for the procedure. In such cases the collection of epididymal sperm postmortem may be a good alternative. Epididymal sperm can be collected from a number of species, but the numbers of insemination doses that may be produced per male differ among species. For example, rams have quite high semen yield, in terms of numbers of doses per male (e.g., Ehling *et al.,* 2006). In addition, epididymal ram sperm has been shown to have good freezability and also good fertilizing ability in cervical as well as laparoscopic inseminations. Details on the various semen collection procedures are presented in Chapter 8.

Techniques for the use of stored semen via AI vary according to species. Both surgical and non-surgical procedures have been developed for most major livestock species. The amount of training to become proficient in each technique varies from species to species as does the rates of success. Table 4.1 gives an overview of parameters for surgical and non-surgical AI of various farm animal species, including expected pregnancy rates. The AI approach to be used and the corresponding pregnancy rates should be considered when determining the number of doses of semen to be conserved (See Chapter 6).

Table 4.1. Comparison of non-surgical and surgical artificial insemination (AI) in farm animals when using frozen-thawed semen.

Parameter[a]	Cow	Sheep	Goat	Sow	Mare
Non-surgical AI					
Pregnancy rate (%)	45-75	15-50	20-55	60-90	35-70
Difficulty[b]	1	5	4	2	1
Recommended for use?	yes	yes	Yes	Yes	yes
Surgical AI					
Pregnancy rate, (%)	65-85	55-85	55-85	85-90	60-80
Difficulty	4	1	1	2	3
Recommended for use?	no	yes[c]	No	No	no

[a] Ranges presented are estimated from multiple scientific and in-field sources. The values vary based on expertise of the technicians, optimal animal nutrition and management practices.

[b] Difficulty of the insemination procedure with 1 being the easiest and 5 being the most difficult to perform.

[c] Assuming that experienced surgical team is available to conduct the procedure.

4.2. Embryos

Since the first reports of birth of normal offspring from cryoconserved mouse embryos in 1972 (Whittingham *et al.,* 1972), similar successes have been reported in more than 16 mammalian species, which includes all the major farm animals. For those species for which collection and transfer techniques are available and operational, embryo banking is a very good possibility for preservation of genetic diversity, and the fastest way to restore an original breeding population when needed, including both the nuclear and mitochondrial genetic information. Embryo technology, however, is usually more costly and requires greater technical capacity than gene banking with semen.

Embryo collection and transfer can be practiced for nearly all of the major livestock species, although the relative difficulty and expected success varies according to species. Whereas, live offspring have been reported born from frozen-thawed embryos of most common livestock species, the best success from this cryotechnology has been with cattle embryos. In cattle, cryopreservation of embryos has been successful and is a routine procedure. Both slow-freezing and vitrification protocols (See Chapter 7) are effective (van Wagtendonk-de Leeuw *et al.,* 1997). The success of cryopreservation is dependent on the stage of the embryo; that is, especially good results are obtained with blastocysts. Procedures for cryoconservation of buffalo embryos have largely resulted from adapting techniques for cattle, but fewer resources have been spent on development and refinement and as a result success rates are generally much lower. The possibilities for cryopreservation of sheep and goat embryos are similar to that for cattle (e.g. Fogarty *et al.,* 2000; Rodriguez Dorta *et al.,* 2007) and cryoconpreservation of horse embryos is somewhat less efficient (Ulrich and Nowshari, 2002). Of all the major livestock species, cryopreservation of pig embryos has long been the most problematic, due to extreme chilling sensitivity and the high lipid content of the pig embryos. In fact, still today swine embryos remain the most difficult to freeze and thaw for offspring production. However, recent studies have focused on overcoming these problems and produced successful methods for cryopreservation of pig embryos (e.g. Nagashima *et al.,* 1995; Vajta, 2000) and live piglets have been obtained (e.g. Dobrinsky *et al.,* 2000; Nagashima 2007).

In addition, species differ with respect to the difficulty of collection and transfer and whether surgical or non-surgical collection procedures are preferred. Table 4.2 compares surgical and non-surgical collection for the major livestock species and Table 4.3 compares transfer procedures. The information the two tables should be considered when planning a gene banking programme, both to choose the type of germplasm to store and the quantity needed. In addition to these five major mammalian species, other livestock species for which live offspring have been obtained from cryopreserved embryos include the dromedary camel (Nowshari *et al.,* 2005) and rabbit (Naik *et al.,* 2005). Pregnancies have been reported in llama (Aller *et al.,* 2002), and red deer (Soler *et al.,* 2007).

Table 4.2. Nonsurgical versus surgical embryo collections.

Embryo Collection Type	**Cow**	**Sheep**	**Goat**	**Sow**	**Horse[d]**
Nonsurgical					
Difficulty[b]	1	5	4	3	1
Percent of treated females with ≥1 embryo/collection	85	<20	<35	<35	80
Transferable embryos per collection (n)	4-8	0-3	0-3	0-5	≤1
Collections per year (n)	3-6	1-2	1-3	2-4	4-6
Recommended for use?	yes	no	no	no	yes
Surgical					
Difficulty	5	2	2	1	4
Percent of treated females with ≥1 embryo/collection	85	75	80	95	<80
Transferable embryos per collection (n)	4-8	3-8	4-9	10-25	≤1
Collections per year (n)	3	1-2	1-2	2	3
Post-surgical adhesions	+++	+++	++++	++++	+
Recommended for use?	no	yes	yes	yes	no

[a]Ranges presented are estimated from multiple scientific and in-field sources. They values are based on superovulated donors along with technicians with embryo expertise, optimal donor nutrition and animal management practices.

[b]Difficulty of the procedure with 1 being the easiest and 5 being the most difficult to perform.

[c]Post-surgical adhesions generally dictate the number of surgical collections per female during her life time. The number of surgeries per female may be designated by governmental regulations and/or an institutional review board

[d] Equine embryos >300 microns in diameter rarely produce a pregnancy from a frozen-thawed embryo following transfer.

Table 4.3. Nonsurgical versus surgical embryo transplantation by species

Transfer Type	Cow	Sheep	Goat	Sow	Mare
Nonsurgical					
Success rate[a] (%)	50-80	10-15	10-15	5-10	55-80
Success rate frozen (%)	50-65	<10	<10	<10	10-20
Difficulty[b]	1	5	4	3	1
Recommended for use?	yes	no	no	no	yes
Surgical					
Success rate, (%)	55-80	50-65	50-65	60-85	60-80
Success rate frozen (%)	50- 65	35-65	35-65	25-60	10-20
Difficulty	3	3	3	2	2
Recommended for use?	no	yes	yes	yes	no

[a]Ranges presented are estimated from multiple scientific and in-field sources. They values are based on superovulated donors along with technicians with embryo expertise, optimal donor nutrition and animal management practices.

[b]Difficulty of the procedure with 1 being the easiest and 5 being the most difficult to perform.

4.3. Oocytes

Similar to embryos, restoration of a lost breed or genotype by using cryopreserved oocytes plus semen would not require backcrossing. In the last 10 years, considerable progress has been made with cryopreservation of oocytes. For a long time, IVF rates with cryopreserved oocytes in humans and in other species had been poor due to (1) release of cortical granules, which causes the zona pellucida to become impenetrable for spermatozoa and (2) disintegration of the metaphase II spindle. IVF rates improved since the introduction of intracytoplasmic sperm injection (ICSI) (Palermo *et al.,* 1992).

In the most recent years, both slow-freeze and vitrification protocols (see Chapter 7 for more details on cryopreservation techniques) seem to render excellent results in humans and are considered to work equally well (Porcu & Venturoli 2006), although there may be even more enthusiasm lately over vitrified oocytes (Jain & Paulson, 2006). Less data are available in farm animals than in humans. This may in part be due to species-specific problems, but it may also reflect that there perhaps has been less incentive to develop and use cryopreservation methods for oocytes in farm animal species compared with human. Viable oocytes have been recovered after freezing and thawing in a great number of animal species, i.e. cattle, pig, sheep, rabbit, mouse, monkey and human (as reviewed by Critser *et al.,* 1997) and in goat (Le Gal, 1996), horse (Hochi *et al.,* 1996), and buffalo (Dhali *et al.,* 2000). Successes have been reported as to post-thaw oocyte maturation, fertilization, and embryo development in a number of species. Live-born young from embryos produced from cryopreserved oocytes have been reported in cattle (e.g. Abe *et al.,* 2005 and Otoi *et al.,* 1995) and horse (Maclellan *et al.,* 2002) as well as several model species. Freezing oocytes of avian and fish species is not successful, largely because of the large size, the high lipid content, and the polar organization (vegetal and animal pole) of bird and fish ova.

In vitro production (IVP) of embryos with fresh oocytes and fresh or frozen semen and subsequent cryopreservation of embryos is an alternative option that may be considered and, in fact, may be more feasible for most species at the present time (See Section 4.7.1).

4.4. Somatic cells

Somatic cells (e.g. skin fibroblasts) can be readily cryopreserved. Collection of suitable somatic cells is straightforward. Cryopreservation protocols for somatic cells are relatively simple and do not require controlled-rate freezing equipment. This means that establishing the collection is easy and cheap, which can be a very attractive option for gene banking, especially for countries with many local breeds and/or limited resources. Viet Nam, for example, has adopted cryoconservation of somatic cells as a primary component of its AnGR conservation programme (See Box 4.1). The complexity and costs are much greater, however, for using the material when needed. Utilization involves cloning and culturing the cells after thawing (or before), reprogramming of the nuclei, collection of oocytes by ovum pick-up or from slaughtered animals, culture and *in vitro* maturation of the oocytes, enucleation of the oocytes, transfer of the somatic nucleus to (or fusion of the somatic cell with) an enucleated oocyte, culture of the resulting embryos, and finally, transfer into recipients of the same species. The use of nuclear transfer (NT) means that the original mitochondrial genotype is lost. In addition, cloning involves some ethical issues, as there are lingering concerns about the welfare of cloned animals and the safety of their food products. Therefore, many countries have not yet approved the consumption of products from cloned livestock animals.

Box 4.1. Cryobanking of Somatic Cells in Viet Nam

Somatic cells offer a convenient solution for collection and long-term storage of AnGR under adverse conditions with limited infrastructure. As such, cryobanks of somatic cells can serve as a backup source of genetic material for regeneration of endangered livestock breeds. With the assistance of researchers from Germany (Groeneveld, *et al.*, 2008) a protocol for collection and banking of somatic cells was developed and tested in Viet Nam. The procedure was developed to be applicable across all mammalian livestock species, by performing sample collection by using specially designed ear taggers (often used for disease diagnosis or DNA testing) with an integrated tag and vial system that cuts a skin sample from the ear and deposits it in the vial while simultaneously attaching the ear tag. The researchers also used a hand-held global positioning system to capture geographical coordinates and a digital camera to take individual photos of each animal. Breed information was also recorded. The procedure was tested in a pilot study that collected and cryoconserved samples from six local Vietnamese populations of pigs (3 breeds), goats (2) and sheep (1). The materials for the gene banking (e.g. ear taggers and tags, liquid nitrogen tanks, and computer) required an initial investment of around €3000, whereas the variable costs to conserve 50 samples from one breed (ideally, 25 females and 25 males) was less than €1000. Sampling was undertaken by the Institute of Agricultural Sciences of South Viet Nam with the assistance of local government agencies and collection of the 300 samples (6 breeds × 50 animals) was completed in 2 months. After three months, viability of the cells was tested by culturing cells from a sub-sample of 23 vials; 100% viability was observed. Although local scientists do not have extensive experience in cloning, the banked cells ensure that the six breeds can be reconstituted in the future if the *in vivo* populations are lost. Somatic cell cryoconservation has since been extended to other species and breeds[1].

[1] http://www.angrin.tlri.gov.tw/cryobanking_2008/7_Le-Thi_Thuy.pdf.

Live offspring have been obtained from cloned embryos in a number of livestock species including sheep, goats, cattle, water buffaloes, pigs, horses, mules, camels, deer, rabbits, cats and dogs. However, for cattle and sheep only a small proportion of embryos produced using somatic cells develop to become live young - typically between <5% - although the efficiency is gradually increasing. A significant proportion of pregnancies are aborted, and full-term pregnancies often result in malformed young. For pigs and horses, greater success rates are reported, with near normal rates of malformed young. Viable litters of cloned pigs are now obtained routinely by transferring large numbers of somatic cell nuclear transfer (SCNT) embryos into each recipient. In fact, a number of companies offer cloning as a commercial service and supply biopsy kits that livestock keepers can use to collect a skin sample and send it to the cloning company for immediate generation of cloned animals or long-term storage for future use. In general though,

the present cloning techniques frequently introduce errors that affect embryonic and fetal development. Costs tend by high for most species, with the possible exception of pigs. On the other hand, on a long time horizon, such as the horizon that might be expected for reconstitution of an extinct breed, increased understanding of nuclear reprogramming is likely to make cloning more reliable and efficient, and thus less costly. Thus, somatic tissue cryopreserved today may be used successfully and efficiently in the future. Therefore cryobanking of ear or other skin tissue can be therefore considered as a cheap method of ensuring the conservation of valuable genotypes for the more distant future.

4.5. Cryopreservation of DNA for genetic analyses

DNA carries the genetic information from the male and female, which will be transmitted to the next generation by syngamy of two gametes. This information is coded by units of DNA termed genes, which can be identified, mapped onto segments of the chromosomes and isolated through basic molecular procedures. Researchers are now using stored somatic cell nuclear DNA to conduct various genetic analyses of animal populations

In the future, the characterization of genes on various chromosomes will likely represent an integral part of conservation (Allendorf *et al.,* 2010). One of the more immediate applications of DNA lies in its ability to determine the underlying genetic structure of populations. Various methodologies (e.g. restriction fragment-length polymorphisms, microsatellites, single nucleotide polymorphisms, and direct sequencing, etc.) are routinely available to rapidly screen populations for genetic variation, providing a level of details previously unimaginable. This knowledge of the partitioning of genetic variability has a role in making informed conservation decisions, and has already been used to set conservation priorities in natural species. Furthermore, such information can provide details on the levels of genetic admixture within a breed, or on the levels of introgression from other populations or breeds, thereby providing an indication of the level of genetic erosion through crossbreeding (Bradley *et al.,* 1994). These uses are taken up in more detail in the accompanying *FAO Guidelines on the Molecular Characterization of Animal Genetic Resources*.

Also, the transfer of genes from one individual to another has attracted a great deal of interest among researchers and pharmaceutical companies. Although progress in the area has been considerable, much of the initial promise, especially for livestock species, has not yet been realized. Some difficulties include the ability to regulate gene expression at the correct stage in development and gene incorporation into the correct tissues. As many animal production traits of interest are modulated by multiple gene expression rather than by a single gene, their cohesive regulation is complex and remains to be determined. How functionally related, yet disparate genes might be transferred into an individual and regulated in a manner compatible with functional body activities is still unclear.

In the very long term, regeneration of an organism from nothing more than its DNA may eventually be possible. In fact, with refinement in DNA synthesis, regeneration of an individual may theoretically be feasible with simply the DNA sequence. However, such *in silico* conservation would require a great deal of technological advancement and can currently be recommended as only a complement to established *in vitro* and *in vivo* approaches.

4.6. Choice of the genetic material to store

The type of genetic resource material to be preserved in the gene bank may depend on the purpose of the gene bank, e.g. whether the gene bank is intended to serve to support *in vivo* breeding and conservation schemes (Sonesson *et al.,* 2002) or is simply designed to save and preserve present day biodiversity for “eternity” or at least for improbable emergency situations in the finite future. In the former case, semen (and embryos), which can be updated periodically and can also be regularly taken from the gene bank and be readily used in the field, are the most practical options. If gene banking is intended to save and preserve present day biodiversity for “eternity”, methods allowing cheap and fast collection of as many species and breeds as possible

are desirable. Wherever financial resources and existing expertise and facilities are available, embryos are usually the best choice. When such resources are not available, collection and cryopreservation of somatic cells should be considered as a reasonable option (Groeneveld *et al.,* 2008). Table 4.4 summarizes the characteristics and advantages and disadvantages of cryoconservation via different types of germplasm.

Table 4.4. An overview of some characteristics of several ways to cryopreserve genetic diversity (adapted from Woelders *et al*., 2003)

Characteristic	Semen	Semen and oocytes	Embryos	Somatic cells
Samples needed to restore a breed[1]	2 000[2]	2 x 100 of each	200	Tissue[3] from ≥60[4] animals (30♂ and 30♀)
Backcrossing needed	Yes	No[4]	No	No
Mitochondrial genes included?	No	Yes	Yes	No
Collection possible for livestock species?	Mostly, not always	Yes, some species. Operational for bovine	Yes, some species. Operational for bovine	Always
Cost of collection	$$	$$	$$$$	$
Cryopreservation possible?	Yes	Still in experimental stage	Bovine, Horse and Sheep are OK. Promising in Pigs. Poultry is impossible	Yes
Utilization	Surgical or nonsurgical insemination backcrossing for ≥4 generations	IVM/IVF[5] followed by surgical or nonsurgical ET	Surgical or nonsurgical ET	Transfer in enucleated oocytes, followed by surgical or nonsurgical ET
Current feasiblity	High	Medium	High, depending on available resources	High for conservation, Low for regeneration of offspring. Future development seems likely.

[1] See Chapter 6 for more specific details.

[2] Dependent on species, reproductive efficiency, and other factors.

[3] Sections of at least 2.5 × 2.5 mm

[4] To obtain a final population of ≥50 animals (25♂ and 25♀), while accounting for failures in cryopreservation and cloning

[5] Yes, if only oocytes are stored.

[6] IVM = *in vitro* maturation, IVF = *in vitro* fertilization, ET = embryo transfer.

4.7. Advanced procedures with current opportunities in cryoconservation

Ongoing research in the biology of gametes and embryos will likely create new methods of recreating individuals from frozen material (see Holt, 1997; Gilmore *et al.,* 1998; Holt & Pickard, 1999; Wildt, 2002; Woelders *et al.,* 2003; Gosden, 2005; Johnson, 2005). The subsequent subchapters describe options that can already be considered today when performing cryobanking. These approaches generally require a great deal of expertise, however, and/or are only applicable in a limited number of species. Nevertheless, similar to SCNT, they are likely to undergo future

development to increase efficiency and decrease costs and thus their potential use may impact decisions on the type of germplasm stored as well as the method for preservation.

4.7.1. In vitro *fertilization with frozen-thawed semen*

Initial IVP utilized oocytes collected from slaughterhouse ovaries. This worked well during early experimentation, when large numbers of immature oocytes were necessary to develop *in vitro* laboratory procedures and to train technical people. In the 1980s it was proposed that the application of IVP in animals would likely be used with genetically valuable farm animal seedstock and possibly of preserving diversity in endangered exotic animals (Loskutoff *et al.,* 1995).

The first frozen-thawed IVF embryo-derived calves were produced in the United States (Zhang *et al.,* 1993). Live animal oocyte collection from live donors and IVF procedures became commercially available to dairy and beef cattle producers in the early 1990s (Bousquet *et al.,* 1999). With thousands of bovine offspring produced worldwide, IVF with frozen sperm is now used routinely in commercial cattle embryo transplant units. Success rate is decreased, however, if the embryos have been frozen and then thawed prior to recipient transfer. Although years of research has been conducted in this area, today IVF methodology is still being tested and fine-tuned for both dairy and beef cattle.

IVF is a multi-step process that requires a well-equipped laboratory and a skilled technician. As the name implies, the IVF procedure involves harvesting the oocytes from the donor's ovaries and fertilizing them *in vitro*. The resulting embryos are held in an incubator for 7 or 8 days and then frozen or transferred nonsurgically to recipient females at the same stage of their estrous cycle. With improvements in oocyte maturation and sperm maturation methods, IVF rates of bovine oocytes are expected to be >85% (e.g. Zhang *et al.,* 1992). The pregnancy success rate for good quality IVF-derived frozen bovine embryos is expected to range from 35 to 50%.

Identifying appropriate and efficient *in vitro* culture systems for IVF-derived embryos seems to be one of the major bottlenecks to IVF application in other farm animal species at the present time. Although the first IVF offspring were reported in sheep and swine (Cheng *et al.,* 1986) and in goats (Hanada, 1985) in the mid-1980s, the IVF procedures developed have not been accepted by the commercial livestock industry primarily because of the high cost of production.

IVF in the mare has not developed to the level for in-field use as expected. Although several offspring were reported in France using IVF in the mare in the early 1990s, repeatable IVF protocols are presently not available. Attempts by many others to produce IVF foals have not been successful making it clear that more research was needed before progress could be made in horses. The reasons for the low success rate of equine IVF remain unclear. Equine oocytes have a thick zona pellucida compared with other species and *in vitro* maturation (IVM) of these oocytes takes longer than for other domestic farm animal species (Hinrichs *et al.,* 1993). The thick zona pellucida of IVM oocytes may act as a barrier to sperm prepared *in vitro*. The potentially altered zona pellucida found in IVM oocytes, in addition to less than adequate sperm cell preparation, likely contributes to poorer than expected IVF embryo production rates in the horse.

It is proposed that IVM and *in vitro* sperm zona penetration are primary problems hindering IVF development in farm animals (e.g., horse), other assisted reproductive technologies, such as zona drilling, zona renting, subzonal sperm injection, intracytoplasmic sperm injection are now under investigation for use in farm animals (Gao *et al.,* 2004; Guerrero,et al., 2008; Chiasson *et al.,* 2010). There is still much to be studied and learned in the use of assisted reproductive technologies to maximize reproductive potential in genetically valuable animals (Hansel and Godke, 1992).

Attempts have been made to use IVF procedures to cross-fertilize different bovine species. In a recent study, an attempt was made to use African buffalo sperm for IVF of domestic cattle ooctyes (Owiny *et al.,* 2009). Although fertilization did occur with some cattle oocytes, very little development occurred thereafter.

With the further development and fine-tuning to improve the repeatability of oocyte retrieval and culture of oocytes before or after cryopreservation, IVF procedures will likely become very competitive with or superior to conventional embryo collection and cryopreservation for gene banking of AnGR, due to the cost and labour advantages .

4.7.2. Intracytoplasmic sperm injection (ICSI)

Studies by research groups in many countries have been attempting to develop techniques to produce offspring from microinjection of sperm cells into unfertilized ova (e.g. Uehara & Yanagimachi, 1976; Markert, 1983). Using this technique, the premise was that the ovum of the female would be activated by a microinjected sperm. The first ICSI experiments in mammals were conducted in rodent species, however, results were variable.

The first live offspring was produced by ICSI into the ooplasm of rabbit ova (Hosoi *et al.,* 1988). IVF and normal cleavage of IVM oocytes were first reported for cattle following ICSI with *in vitro* capacitated sperm (Younis *et al.,* 1989). The first transplant offspring in farm animals (live calves) from ICSI of bovine oocytes was reported in Japan (Goto *et al.,* 1990). Varying levels of success have been reported since.

Among livestock, ICSI is most advanced for horses. Among the first successes were pregnancies produced using oocytes from abattoir horse ovaries (Squires *et al.,* 1996), nonpregnant mares (e.g. Meintjes *et al.,* 1995; McKinnon *et al.,* 1998) and pregnant mares (Cochran *et al.,* 1998 and 2000). Today, the ICSI procedure being used routinely for mares with low fertility via conventional means.

Although ICSI is quite successful with horses, the technology is not yet ready for routine use in most livestock. However, similar to conserving somatic cells for cloning, if germplasm is to be conserved for long-term storage, one can probably afford to store semen and wait for the technology to be developed until it has to be used. Once the technology is developed to the point to become routine, ISCI has the potential to dramatically increase flexibility. For example, mistakenly thawed bull semen could be refrozen thawed again and then used for ICSI. Recently, a calf has been produced form ICSI with frozen-thawed bovine epididymal sperm (Guerrero *et al.,* 2008). ICSI also holds the potential to be used with freeze-dried semen, which would eliminate the need for cryoconservation in LN2 and greatly facilitate *ex situ* conservation of AnGR in countries where sourcing of LN2 is a problem.

Figure 4.1. Intracytoplasmic sperm injection (ICSI) procedure (courtesy of R.A. Godke).

4.8. Future prospects for cryoconservation

4.8.1. Cryopreservation of ovaries and other gonadal tissue

Cryopreservation of ovaries could be another way to conserve valuable AnGR. In the human, ovary banking is routinely used to prevent loss of fertility during cancer treatment of women. In addition, it is considered as an effective method to cryobank the thousands of strains of mice in centres for laboratory animals and research centres in general.

Cryopreserved ovaries of parts of ovaries may be used as a source of oocytes. Oocytes may be harvested from heterotopically grafted ovaries to be subsequently used in an IVF procedure to produce embryos. The embryos must then be transferred to a recipient animal. Alternatively, cryopreserved ovary tissue or whole ovaries could be grafted orthotopically in a recipient animal in order to restore fertility in that animal. This animal can then be mated and will produce offspring carrying the ovary donor genotype.

The production of live offspring after orthotopic transplantation of sliced frozen mouse ovarian tissue was first reported more than fifty years ago. Since then, there have been reports of successful orthotopic allografting of mature and juvenile mouse ovaries to recipient mice (e.g. Candy *et al.,* 2000). Recently, Japanese quail ovarian tissue was successfully frozen, thawed and transplanted to young chicks, which then subsequently produced live offspring after mating (Liu *et al.,* 2009). Restoration of fertility after grafting cryopreserved oocytes was also achieved in larger animals, e.g. using vitrification of (hemi) ovaries in sheep (e.g. Bordes *et al.,* 2005). Song and Silversides (2006, 2007a) have successfully obtained offspring from procedures involving cryopreservation and subsequent transplantation of both male and female gonadal tissue in poultry.

Additional research this technology would offer new possibilities for future animal production systems but requires additional research and development, especially for mammalian species. Further improvements are needed in the cryopreservation procedure and the grafting technique but, similar to cloning and somatic cells, advancement in these techniques could be expected to occur in the interim if the ovaries are used for long-term storage. Nevertheless, even with such technological advances, cryopreservation of ovaries or ovarian tissue may be less efficient than cryopreservation of embryos for *ex situ* conservation of genetic diversity of farm animals. First, either laparoscopy or sacrificing of the donor animal would be needed to obtain the ovaries. Second, to make use of the cryopreserved material to produce offspring, surgical expertise and facilities are again required for the grafting of the thawed cryopreserved ovaries into recipient animals.

Like oocytes, the storage of ovaries would require either complementary cryopreservation of semen or the application of a backcrossing strategy similar to that when only semen is used, but by using male founders or semen from another breed. Such a strategy would result in the loss of genetic material on the Y-chromosome of mammalian species, however. Given the generally lower cost and greater ease of preserving semen, preservation of only oocytes or ovaries would be logical in only specific instances.

4.8.2. Embryonic Stem Cells (ESC)

ESC are defined as undifferentiated embryonic progenitor somatic cells that have been cultured *in vitro* and frozen for later use. Today, such cell lines have been established in laboratory species and are being used to generate transgenic animals carrying cross species or tailored genes. The advantage of these cells is that they can be frozen and later thawed then multiplied through numerous cell cycles. In the animals species (i.e. mice), where true ESC have been identified, they are obtained relatively easily from cultured young embryos (inner cell mass of the blastocyst stage) or early stage germ cells (e.g., primordial germ cells) and can be kept frozen for future use.

If ESC are introduced into an activated enuculated oocyte or possibly an embryo at the beginning of its development they can influence cell subsequent development in various body tissues throughout life. ESC could thus be potential vectors for the transmission of genetic

characteristics. However, even with an intensive research effort in recent years, there is at present no convincing evidence of the existence of true ESC among livestock species. If these cells and adult somatic stem cells could be used in domestic species with a reasonable rate of success, then this new stem cell technology would be a useful tool in cloning, clinical veterinary medicine and even for the preservation of genetic diversity.

4.8.3. Spermatogonia

These cells reside within the basal layer of the seminiferous tubules of the testis and have the capacity to give rise to spermatozoa. Beginning before puberty and continuing in the adult animal, spermatogonia undergo continuous replication, thereby, maintaining their number in a process known as stem cell renewal. It has been shown earlier in mice (Brinster and Zimmermann, 1994) that these stem cells, when isolated from testes of donor animals, can be processed and used to repopulate another testis without evidence of immuno-rejection.

Kimura and Yanagimachi (1995) have reported the development of normal mice from oocytes injected with secondary spermatocyte nuclei. Recently, frozen-thawed testicular tissue from day-old chicks was transplanted to host chicks and resulted in live offspring from the donor tissue sperm (Song and Silversides, 2007b). This approach could potentially be used to pass genetic material from one generation to the next, and when frozen spermatogonia could be a means for saving genes from the male animals for cryoconservation of AnGR.

4.8.4. Primordial germ cells

Although efforts have been made over the years to produce gametes and offspring from primordial germ cells (e.g., Tsunoda *et al.,* 1989, Chuma *et al.,* 2005), it has been only recently that increased success has been reported in fish and in birds. In chickens, primordial germ cells usually migrate to the gonadal ridge via the blood stream between days 4 and 6 of incubation. During this migration stage, the primordial germ cells can be harvested from the blood of one chick embryo, cultured and transferred to other developing chick embryos, resulting in germline transfers (Etches, 2010).

Male or female primordial germ cells of quail have been successfully transferred into chick embryonic gonads (e.g. Ono *et al.,* 1996). Germline chimeras have also been reported with host quail that have subsequently produced live offspring from the donor quail germ cells (Kim *et al.,* 2005). Using germline transplantation, live offspring have been produced by surrogate birds from other avian species (e.g., pheasant) (Kang *et al.,* 2008). This research area holds promise for cryopreservation systems in the future.

4.8.5. Parthenogenetic and IVF embryo reconstruction

Only limited embryo development has resulted from various attempts over decades of research on parthenogenetic embryo production. One approach to attempt to recover female germplasm used embryo micromanipulation techniques to make chimeric embryos, each with a combination of material from a parthenogenetic bovine embryo (from one breed) and an IVF bovine embryo (from a second breed) (Boediono *et al.,* 1999). The reconstructed chimeric embryos were then transferred to recipient cattle and resulted in live offspring, each of which had a chimeric genotype (parthenogentic and IVF) and exhibited the distinct coat colour patterns of both breeds (phenotype). In a breed or species with no males remaining, one could attempt save the germplasm by producing a female offspring from reconstructed ovaries in an effort to produce oocytes from their chimeric ovaries.

4.8.6. Gametes derived from embryonic stem cells

Mouse oocytes have been derived from embryonic stem cells (Hubner *et al.,* 2003). If this methodology can be further developed for livestock species, it could have important potential implications for oocyte and embryo cryopreservation.

4.8.7. Cloned embryo reconstruction

Poor placental development has, in part, been blamed for the loss of cloned pregnancies during early and late gestation in recipient cattle. It has been proposed that if one could exchange the placental tissue (embryonic trophoblast) of the cloned embryos (embryo reconstruction), one might enhance normal fetal development *in utero* and thus, produce more viable cloned bovine offspring. Thus, efforts are underway to use embryo reconstruction of IVF-derived and SCNT-derived cattle embryos to improve nuclear transfer production rate (e.g., Murakami *et al.,* 2006).

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5. PHYSICAL STRUCTURE OF THE GENE BANK AND REQUIREMENTS AND COSTS TO ESTABLISH SMALL, MEDIUM AND LARGE GENE BANKS

Gene banking operations and facilities are likely to vary substantially from country to country, in terms of both the size and capacity of the bank and the types and amount of equipment needed. These factors are dependent upon the quantities of germplasm to place in the gene bank, which will be based upon the objectives of the gene bank, the range of species and breeds be conserved and the financial resources available for the conservation program. For the purposes of this manual, the types of facilities and equipment need to operationalize a gene bank are subdivided into three sizes: small, medium and large.

In many situations the elements needed to establish a gene bank will already be in place. For example, some countries have state-owned and operated AI studs or possibly an existing gene bank for plants or wild animals. If such operations are already available, the AnGR gene bank can be incorporated into these programmes. Certainly such a combination would facilitate the development and collection of some livestock species. However, additions to facilities and equipment may be necessary and health and sanitation issues must be considered, especially for any facilities that might be shared between wild and domestic animals.

For all facilities there are common features necessary for the smooth functioning of the gene bank, regardless of size. These are the physical plant (i.e. the actual building and other structures), durable equipment, security arrangements, centralization and accessibility. In addition, specialized human resources are a critical component for successful gene banking activities.

5.1. The physical plant

The building housing the gene bank will vary dramatically depending upon the total size of the facility and its operations. For example, if animal and plant gene banks are housed together, the building requirements will be different than for a single-purpose facility for AnGR. For livestock, specific components of a single- or multiple-use facility, such as the laboratories for germplasm acquisition, processing and cryopreservation do not need to be located in the same physical space.

In developing the gene bank (including long term storage room(s) and laboratories) the services of a professional architect will usually be needed. Clearly, such professional assistance will be required to develop specific blueprints and construction plans if no physical structure exists. In addition, in some instances a physical structure may exist but the available space may not be suitable. Therefore, re-design and some refurbishing will be necessary and such processes will usually require the employment of an architect and/or engineer.

The actual collection and banking of animal germplasm comprises of three main activities: 1) collection of the germplasm, 2) processing and freezing of the germplasm, and 3) storage of the germplasm. In general, although all of these activities may be undertaken at the same location, they are distinct and each requires its own separate facilities. In theory, a gene bank need only involve a place for storage of germplasm, assuming that the germplasm is provided from elsewhere. In most situations, a gene bank will have the infrastructure for at least two, if not all three activities.

5.1.1. Animal housing and collection facilities

Many gene banking operations will find it practical and convenient, or even necessary, to have a dedicated facility to hold animals while their germplasm is being collected. Depending on the species and germplasm to be conserved, collection of a significant quantity of germplasm will be impossible during a single intervention with a given animal. For example, even with superovulation, collection of embryos from cattle will yield only a few embryos per collection, so the process will need to be repeated several times per donor. The process of superovulation generally requires administration of a regime of hormones over the course of several days prior to embryo collection, so having the donors in a central facility is practical. For semen donors, training the males for collection will usually increase yield and quality. For countries aiming to adhere to the standards for export of the World Organization for Animal Health (OIE - Office International des Epizooties), holding facilities will be needed to quarantine animals prior to collection and monitor health after collection (see www.oie.int).

5.1.1.1. Building materials

When constructing the facility the choice of materials for internal and external construction is critical. The building must be strong enough to withstand environmental challenges specific to the locale as well as the normal wear and tear and abuse inflicted by the animals housed at the facility. Likewise, the internal surfaces must be resistant to endure the physical abuses of the animals and must also be capable of being sanitized. Therefore, the choice of the materials must be impermeable to water and able to withstand repeated cleaning with sanitizing chemicals. The flooring must be coarse enough so that animals can maintain stable footing when being collected (e.g. bulls when mounting a teaser animal).

5.1.1.2. Multiple buildings

Prevention of the spread of disease among the animals held in the facilities and to the germplasm stored in the bank is critical. For this purpose, the facility should have a system for quarantine of all incoming animals. If possible, multiple buildings should be utilized at a collection facility. Then a quarantine system can be set up so that an “all in/all out” policy can be implemented. Once one group of animals leaves a building, the building can be cleaned so that the new group of animals can be brought into an empty, sanitized building and quarantined. Once the quarantine is lifted they can then proceed back and forth to the separate collection building, but may never again enter a non-sanitized housing unit. Animals with questionable health must always be

housed separately from healthy animals and never intermingle directly or utilize common spaces such as the collection facility. In addition, if the facility is planned to hold several species, each one have to be kept in different and separated buildings.

5.1.1.3. Environmental control

The facility should be designed so that the air flows in one direction through the building. This air-flow scheme limits the "dead spaces" that may be created by air flowing in multiple directions within the facility. Furthermore, it ensures that only fresh air will enter the building and air laden with animal by-products (methane, urea, etc) will be vented outside, thus minimizing the load of contaminants and improving the health of the animals being housed there. Proper ventilation will also help maintain the environment at the proper humidity. Temperature control is another important element. The need for heating and/or cooling systems must be considered in the context of the natural climate in order to maintain the indoor temperature within the range of thermal comfort zones of the species being housed inside. In particular, the animals have to be protected against higher temperatures, which can adversely affect sperm production, especially in pigs. In addition, the intensity of light and the periods of light and dark may also have to be controlled in order to meet the physiological and ethological needs of the animals.

5.1.1.4. Biosecurity

Biosecurity is undoubtedly one of the most important concerns of a gene bank manager, and to guarantee it, the following principles should be considered:

1. Location of the facilities: It is essential to carry out a detailed study of the possible location before starting the construction of a new gene bank. If possible, the facilities should be situated in a low-animal density area. The animal holding buildings should be situated at least 3 km from other farms or similar sources of biological risk, whereas the distance from the nearest main roads or railways should be at least 1 km, especially if the nearby roads and railroads are frequently used for the transport of livestock.
2. Perimeter fence: The installation must to have a perimeter fence to prevent the entry of non authorised persons and other domestic or wild animals.
3. Animal loading bay: This structure poses a great risk. The loading bay for arrival and departure of donor animals should be situated outside the perimeter fence and some distance from the animal housing units. Also, trucks bringing feed to the facilities should unload in storage and transfer areas outside the perimeter fence.
4. Clean-Dirty Areas: The facility must be clearly divided in two physically separated areas: the dirty area (buildings for animals, collection pens, storage areas for waste materials, feed, etc.) and a clean area (laboratories, instrumentation rooms, germplasm storage facilities and offices). If these two facilities are in the same building, only one indirect connection between the clean and dirty areas should be allowed, ideally through a sanitary air lock in the wall between collection pens and laboratories. Finally, locker rooms with changing facilities and showers should be located between the two areas.
5. Staff: The members of staff should work exclusively in the gene bank and not have contact with other farms or facilities with the same biological risk. The clothes, shoes and footwear used in the laboratories should be stored in the changing facilities and not used or otherwise taken outside.
6. Waste management: the waste management should safe and adequate to reduce the risk of disease transmission, storage facilities from where waste will be loaded and transported away must be outside the perimeter fence.
7. Pest control: Common pests include rats, mice, bird and flies. The control of rats and mice is greatly facilitated by adequate construction and maintenance of the facilities, particularly the prevention of access to feed and water. Traps and rodenticides are alternatives for control if problems are discovered. Bird proofing nets in windows and

other openings will limit the entry of birds, although their control in open-sided buildings or outdoors pens is very difficult. Regular cleaning of pens, strategic use of insecticides and proper ventilation are important elements in the control of flies.

8. Dead animals: Even with the greatest care and precaution, death of the occasional donor is likely to occur. Carcasses should be taken away as quickly as possible and stored outside of the perimeter fence until they are collected. Access by stray or wild animals and birds must be prevented.

5.1.1.6. Working, handling and containment pens (indoor and outdoor)

Animal pens should be constructed with the following factors in mind:

1. Size: The pens should be appropriate for the animals' size and be large enough so that minimal handling of animals is required when moving them into and out of the facility. For example, if sheep or goats are kept in pens that were designed for cattle then a large amount of time could be spent on herding or coaxing the animals to the collection site, which will create stress on the animals and decrease the quality of the germplasm samples collected.
2. Individual pens: Male donors should be kept in individuals pens, but they should have visual contact with other animals. The objective of this measure is to avoid aggression between males and subsequent injury but to still allow the development of social relationships that help reduce stress.
3. Materials: High-quality, durable pens should be constructed so that they can handle the repeated stresses of animals.
4. Automatic systems: When financially feasible, the holding pens should ideally have automatic systems to supply water and feed to animals, and to eliminate waste, in order to reduce the human contact with them and the subsequent stress.
5. Safety: Pens should be free from sharp edges or jutting angles that could result in injury to animals or their handlers. Pens should be constructed with escape routes for handlers in the case that animals become violent.

5.1.1.6. Example animal holding and collection facility

Figure 5.1 is a diagram of a basic facility designed for housing multiple types of animals, such as several rams and 20 to 30 roosters, and for collecting and processing semen samples and embryos or other genetic materials. The freezing and storage rooms will ideally be physically separated from these animal housing and handling facilities for sanitary reasons. The facility is equipped with indoor and outdoor areas for the larger animals, which enable the animals to be moved with minimal handling to a collection area. Also included are three rooms which are self-contained within the facility; the small animal room, the storage and feed room, and the sample preparation room. Having these rooms self-contained allows the facility to have "micro-environments" to fit the needs of the specific room; for example, the small animal room will be required to have a daylight regimen, the feed room needs to be contained to eliminate the potential for rodent infestation, and the sample preparation room needs to have a system for environmental and temperature controlled to decrease the potential of cold-shocking semen samples.

The airflow of the facility is also illustrated with the dotted lines and arrow. As noted, the small animal room is separated from the remainder of the facility (other than an airlock for passage of samples to the preparation room) to minimize the spread of chicken byproducts (e.g. faeces, feathers, etc.) therefore, this room has its own air handling unit which provides fresh air and an exhaust system. Likewise, the large area of the facility has its own air handling and exhaust system for the same reasons. The air handling system of the sample preparation vents into the larger facility and is this removed by a common exhaust system.

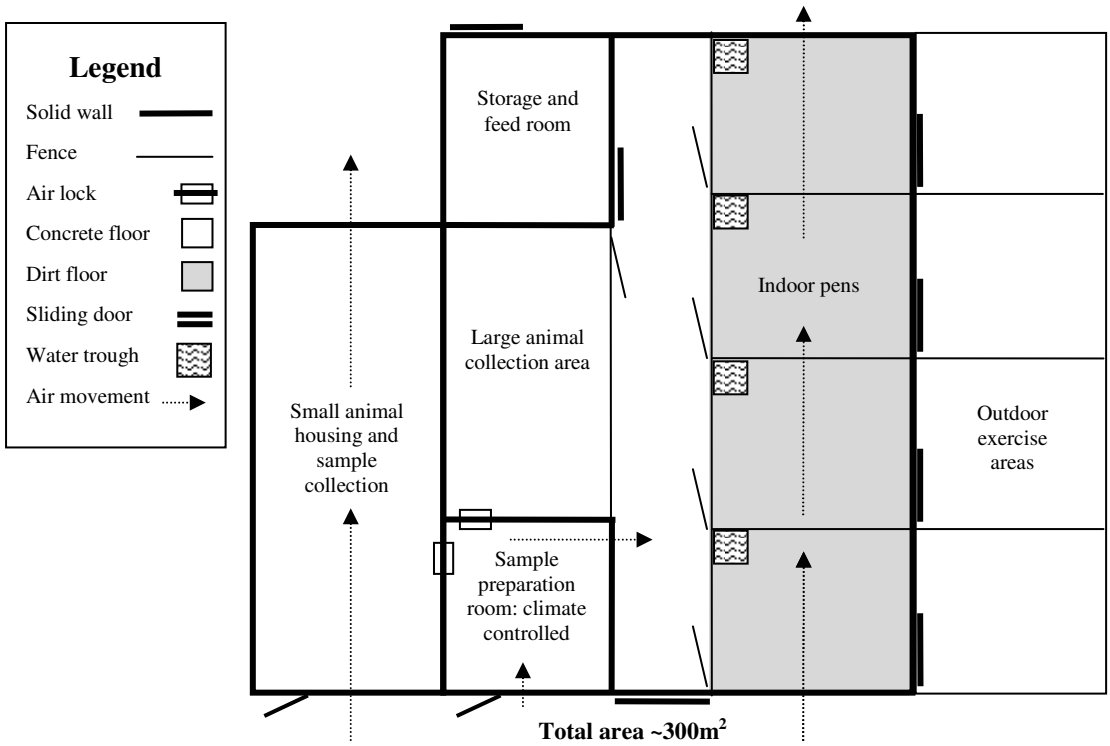
For biosecurity and general safety, several practices can be followed in a facility similar to that in Figure 5.1, which includes only a single animal- housing building. An all in/all out procedure can

be followed, such that a group of donors arrives and leaves together, with the building cleaned and left unused for a period between each group. Different species can be alternated in this system, so that multiple species are not present together. Male animals can be housed in separate pens. Finally, animal handling and sample collection and sample preparation can be the respective responsibilities of two different persons, so that the processing technician does not directly contact the animals.

5.1.2. Field collection

In many countries some collection of germplasm in the field is likely to be necessary, even if the country has a complete gene banking facility including infrastructure for animal holding and collection. Field collection will likely be more common in countries with a large geographic area, and single gene bank, because transport of animals to the central facility will be more expensive, and in countries where pastoral or other pasture-based production systems are predominant. Field collection requires particular protocols and advanced planning, as well as some specialized equipment. A recommended practice is to prepare a specific protocol to be followed during field collection and to train technicians responsible for field collection on best practices for off-site collection. The national gene bank at the Canadian Animal Genetic Resources Laboratory for Cryobiology has a specific set of standard operating procedures for field collection of germplasm and those procedures are summarized here.

Figure 5.1. An example of a simple animal collection facility.



5.1.2.1. Equipment

The following equipment and reagents are recommended or necessary for collection and/or freezing of germplasm in field conditions:

- Disposable coveralls
- Disposable boots
- Single-use examination gloves
- Reusable and sealable plastic bags
- Indelible permanent markers

- Paper towels or absorbent paper
- Frozen ice packs
- Shipping container
- Packaging tape and dispenser
- Portable incubator
- Microscope
- Microscope slides
- Haemacytometer
- Semen straws and filling/sealing equipment
- Styrofoam boxes
- LN2 storage tank
- Gloves and tools for LN2 handling
- LN2 Dry-Shipper
- Pre-addressed shipping labels (to the gene bank)

5.1.2.2. Safety

Field collection of germplasm should always be done by a team of technicians, if possible, rather than by a single technician. Interaction with large animals always opens the possibility for injury, particularly for animals in rangeland production systems that may not have regular human contact. Surgery tools and LN2 are also possible sources of injury. Cellular phones should be carried for general communication and to contact emergency authorities if injuries occur. When collection is undertaken in a remote, unfamiliar area, local authorities should be notified in advance and maps and GIS equipment (also for recording sample site) should be taken.

As with collection at a dedicated facility, animals should be restrained in a manner that is safe for both the animal and the technicians and care should be taken to minimize the stress to which donors are subjected. Biosecurity protocols should be followed to prevent the transmission of disease from location to location (see also Chapter 9).

5.1.2.3. Procedures

To maximize efficiency, specific standard operating procedures for field collection should be prepared and technicians should familiarize themselves with the procedures prior to going to the field. Appendix A has an overview of field collection procedures, based on those used in Canada.

5.1.3. Germplasm processing and freezing laboratories

Although the processing and freezing laboratories may be part of the same physical structure as the animal collection and holding facilities, a distinct physical barrier between the two areas must be present, as the processing and freezing area must have a greater level of sanitation. The laboratory should be designed for maximum efficiency. In particular, it should be kept as small as possible to decrease the area to maintain and keep clean and the various workstations should be arranged in a logical order so that germplasm samples move in a progressive order from workstation to workstation.

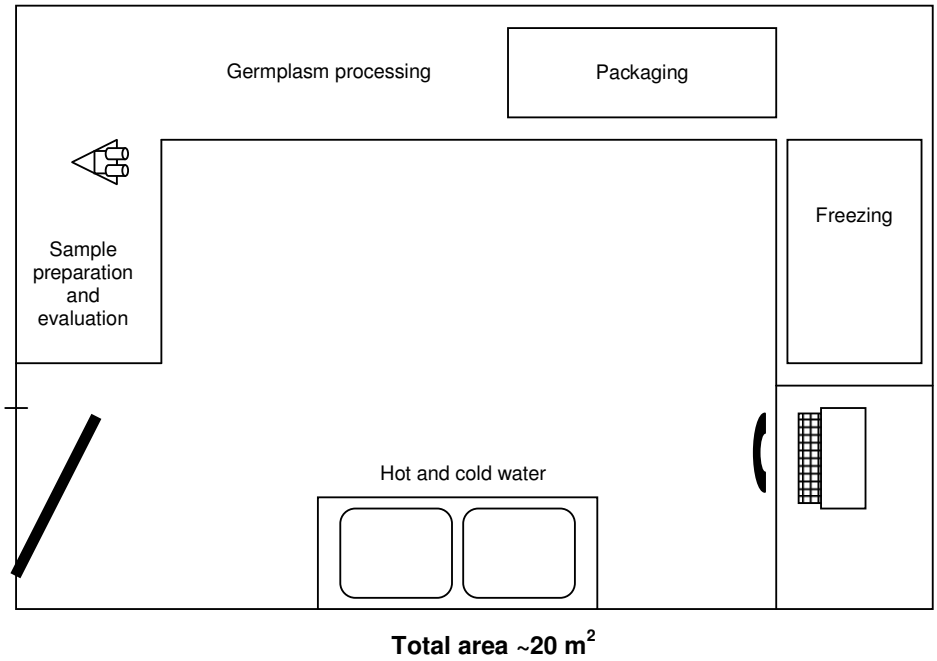
Essential features of the laboratory include the following:

- washable work surfaces, floors (non-slip) and walls,
- sufficient lighting and ventilation,
- hot and cold running water as well as purified water,
- sufficient numbers of electrical sockets for all fixed and portable equipment,
- adequate storage for consumable materials.

Figure 5.2 shows an example of a general layout of a simple germplasm processing laboratory. The space for all activities is efficient, as the design of the various workstations is compact. The process of cryopreservation of germplasm begins at the left in the diagram and then moves step-by-step in a clockwise direction until the samples are finally packaged and frozen. Computing facilities are readily available, both for operation of any software that may be necessary (e.g.

calculating dilution rates and for inputting information about each sample into the central database (see Chapter 10).

Figure 5.2. Example design of a germplasm processing and freezing laboratory.



5.1.4. Long term storage room

In general, the long-term storage room for cryopreserved germplasm can and should be physically separated from any laboratory. Such an arrangement will increase the physical security of the collection. By having physical barriers the potential for unauthorized access to the collection can be better controlled. Many existing gene banks have found this to be a desirable arrangement because then the long term LN2 tanks do not have to be individually locked. That said, gene bank managers may nonetheless consider locking individual tanks by weighing the advantages and disadvantages when establishing their standard operating procedures.

LN2 is a dangerous material (see Box 5.1) and specific precautions must be taken, both in the handling of LN2 and in the design of storage facilities. LN2 can pose a potential health risk, via asphyxiation, to those involved with filling tanks and placing or retrieving samples or simply working in the storage areas if leaks occur. For this reason, in developing long term storage space ventilation design is a critical priority. In general, nitrogen in its gaseous state is heavier than normal atmospheric air, so if LN2 spills the concentration of nitrogen will be greatest close to the floor. Therefore, ventilation ducts need to be placed at or near floor level. Conversely, air inflow ducts need to be placed high in the room (potentially at ceiling level) to help insure that nitrogen gas is forced out of the room through the lower exhaust vents. In rooms where samples are frozen with LN2 there is also a need to have adequate ventilation. In both spaces it is highly recommended that oxygen sensors be put in place, and tested regularly.

Box 5.1. Liquid Nitrogen Safety

Liquid nitrogen is an extremely dangerous substance to handle. Two major safety risks are 1) freezing or "burning" of skin upon contact and 2) hypoxia (lack of oxygen) and respiratory distress. Liquid nitrogen has a temperature of -196°C. Therefore, when working with liquid nitrogen, one must avoid all skin contact. Protective gloves should be worn as well as standard lab coats. Shorts and open shoes or sandals should be avoided; eye protection is recommended. Specially designed "tongs" should be used for handling of straws and containers for storage of

germplasm. The low temperatures alter the physical properties of other substances, so only tools specifically designed for work with LN2 should be used. If contact does occur, one should flood the exposed area with cold water and apply cold compresses. Medical attention should be sought immediately if eyes are affected or if blistering of skin occurs.

To prevent the possibility of hypoxia and respiratory problems, sufficient ventilation is needed in handling and storage areas. Nitrogen vapour is heavier than air, so exhaust fans should remove gas from near the floor. Oxygen meters should be used to warn for the presence of excess nitrogen, and an oxygen concentration of <19.5% is to be considered unsafe. If dizziness occurs, one must evacuate to well-ventilated area. Victims of hypoxia must be moved to safety immediately and subject to artificial respiration and medical assistance.

Transport of LN2 presents special dangers; great care must therefore be taken. Liquid nitrogen is an additional source of risk in auto accidents. Always ensure that tanks are safely secured, and of transport of LN2 in the passenger compartment of a vehicle should be avoided. If this is not possible, one or more windows should be kept open.

5.2. Size and capacity requirements for gene banks of varying sizes

The space required for gene banks can be extremely variable and may change as the mission of the gene bank changes or as the collection grows. It is important to state that the space requirements are relatively minor. Furthermore, the costs of developing the space should be amortized over appropriate depreciation time lines, e.g., ≥20 years.

5.2.1. Small repositories

These repositories have limited space but could easily meet the needs of some country programs. It would be expected that with facilities of this size that up to 500 straws of semen could be processed and cryopreserved in a day.

- Wet lab 20 to 30 m^2
- Ability to cool and refrigerate samples (to 5 to 15°C, depending on the species):
  - Cold room (10 m^2), or
  - Cooler cabinet (2 m^2), or
  - styrofoam box (0.6 m^2)
- Cryotank storage room 20 m^2 (sufficient for approximately 300 000 straws)
- Office for database management 12 m^2

5.2.2. Medium-sized repositories

Larger repositories should have a few more features that would not only increase the amount of germplasm that they can store but also the quantity of germplasm (principally semen) they can cryopreserve in a day (more than 500 straws per day). In such facilities automated equipment for straw labeling and filling are likely to be found and therefore, increase their capacity to process and cryopreserve semen to levels in the thousands of straws per day.

- Wet lab 30 to 60 m^2
- Ability to cool and refrigerate samples:
  - Cold room (10 m^2), or
  - Cooler cabinet (2 m^2), or
  - styrofoam box (0.6 m^2)
- Cryotank storage room 25 to 50 m^2 (approximately 400 000 to 600 000 straws)
- Office for database management (12 m^2)
- Other office space (12 m^2)

5.2.3. Large Repositories

These facilities have substantial capacity to cryopreserve semen, embryos and DNA to meet their national mandates. Compared to medium-sized facilities, equipment is available to increase the quantity of germplasm frozen to greater than 1 000 straws per day. In addition, to the equipment necessary for cryopreserving samples, equipment for analyzing the quality of the germplasm cryopreserved is also found; for example a computer-assisted sperm analysis (CASA) unit and flow cytometers.

- Wet lab greater than 60 m^2
- Ability to cool and refrigerate samples:
  - Cold room (10 m^2 sq ft), or
  - Cooler cabinet (2 m^2), or
  - styrofoam box (0.6 m^2)
- Cryotank storage room greater than 50 m^2 (>500 000 straws)
- Office for database management (20 m^2)
- Other office space (50 m^2)
- Capacity to store excess LN2 or even to make LN2.

5.3. Recommended equipment and estimated costs

The following is a list of basic equipment needed for cryopreserving and storing samples. Box 5.2 has more specific information about the types of LN2 tanks that will be needed. It is important to remember that the purchase of multiple LN2 tanks for long term storage need not occur at the same time, but can be done on a gradual, as-needed basis.

Box 5.2. Liquid Nitrogen Tanks

The number and size of LN2 tanks to be used in the gene bank will depend upon the expected quantities of germplasm to be stored. When purchasing a tank, the capacity will be specified in terms of numbers of straws. Another important factor to be taken into consideration is the quality of the tanks. Choosing to buy a given tank because the purchase price is low may not be the optimal financial strategy in the long term. Higher quality (and thus more expensive) tanks will usually last longer and usually use less LN2. They will also be less subject to leakage and other accidents resulting in the loss of stored material. The value of the stored germplasm will usually far exceed the cost of the storage tank.

Any gene bank will need three types of tanks:

1) Long-term storage tanks,
2) Vapour shippers (for transport of germplasm) and
3) Storage tanks (for storing LN2 itself),

Long-term storage tanks are the most important equipment of a gene bank. Remember that the stored germplasm may be unique and cannot be replaced when lost. The tanks should have low LN2 consumption, because this is one of the major costs of maintaining a gene bank. In addition, it is recommended that individual tanks have electronic monitors that measure temperature and LN2 levels and sounds an alarm if either parameter is outside set limitations. The size of this type of tank can be highly variable depending upon the size of the repository and planned quantity of germplasm to be stored.

Vapour shippers are designed for the safe transportation of germplasm, and contain a hydrophobic absorbent that holds the LN2, repelling moisture and humidity, in order to ensure the maximum holding time (usually from 2 to 3 weeks).

A third type of LN2 tank required is one for storing surplus LN2 for future use in the other types of storage tanks. In addition, this type of tank gives the repository a buffer in the event that regular supplies of LN2 are interrupted. As a result, the gene bank needs to consider what level of security is required in order to determine the size of storage tank needed, based on the frequency

and reliability of deliveries or the production schedule if the gene bank owns a LN2 generation plant.

5.3.1. Basic equipment

- Glassware for holding of semen and measurement of volume
- Water bath
- Microscope (phase-contrast – semen; stereo scope – embryos)
- Centrifuge
- Cooling samples (styrofoam or plastic cooler, cooler cabinet)
- Supply of pure or ultra pure (embryos) water
- Osmometer
- pH meter
- Method for determining sperm concentration (one or more of the following):
  - Spectrophotometer (fixed or portable)
  - Makler counter chamber (or disposable counting chamber), or
  - Haemocytometer
- Temperature measuring devices
- Straw labelling machine
- Straw filling and sealing equipment
- Freezing equipment (manual or programmable)
- Carbon dioxide incubator (for embryos)
- Laminar flow benches (for embryos)
- Dry LN2 shipping tanks
- Long term LN2 storage tanks

5.4. Gene bank security

Germplasm collections can be viewed as a national asset and thereby warrant appropriate levels of security. Building security consists of several aspects including:

- Safety of the germplasm from unauthorized access. This can be achieved by controlling access to the room and the tanks themselves as mentioned above. In addition, gene bank managers should maintain records on which persons have access to the room(s) were collections are stored and when those persons accessed the room.
- Structural integrity. The structure of the gene banking facility should be sufficient to withstand any environmental challenges that are conceivable in the corresponding geographical area (e.g., high winds, earthquakes, ambient temperature extremes, fires, floods). On a micro-level, construction in vulnerable areas such as floodplains, should obviously be avoided.
- Plans and equipment to continue operations in the event of major systems failures. For example, generators for electricity, and alternative sources of LN2 should be considered.

Table 5.1. Estimated equipment costs by gene bank size (estimates in US dollars and 2011 prices).

	Small			
Item	Necessary	Optional	Medium	Large
Long term LN2 tanks	$5 000 to $40 000		$40 000 to $120 000	> $120 000
Shipping tanks	$3 000		$3 000	$3 000
Equipment for straw filling	$500		$40 000	$40 000
Equipment (styrofoam box to programmable freezer) for freezing samples	$200		$200 to $20 000	$200 to $20 000
Microscope	Compound $500		Phase Contrast $5 000	Phase contrast w/ fluorescence $15 000
Centrifuge		$10 000	$10 000	$10 000
Spectrophotometer (fixed or portable)	--	$2 100	$2 100	$2 100
Makler counter chamber	$850		$850	$850
Hemocytometer	$200		$200	$200
pH meter	$1 200		$1 200	$1 200
Osmometer	-	$8 000	$8 000	$8 000
Water bath	-		$800	$800
Total	$11 450 – $46 450	$31 500 - $66 550	$111 350 - $211 150	$201 350 - $221 350

5.5. Centralization and accessibility

To facilitate gene bank development it is beneficial to be located in an area with sufficient infrastructure to ensure smooth and continuous operation. Collection efforts can be enhanced if the gene bank is located near to major highways or airport hubs within the country. Depending upon country conditions, this may or may not be in or near the capital city. An additional consideration in choosing a location is the ease with which germplasm can either be collected or shipped to the gene bank. Thus, areas within a country with the largest populations of livestock are favoured. When the country is large enough to have notable regional differences in climate, situating the gene bank in a relatively colder region can help to minimize the LN2 evaporation and subsequently reduce the cost of maintaining the gene bank.

Another factor in choosing the site for a gene bank is to what extent public and private sector arrangements can be made for germplasm collection and processing and the location of important stakeholders. For example, AI centres may already be operating in a country; in which case the national germplasm programme could develop arrangements with such companies to acquire germplasm from their facility. By formulating such arrangements the national programme can focus attention and resources to breeds and species that lack commercial infrastructure to assist with collection development. As noted previously, existing gene banks for plants or wild animals are also potential collaborators. In addition to aiding in the collection effort, the presence of collaborating institutions may increase the accessibility of needed infrastructure such as LN2.

5.6. Human resources

The number and type of personnel needed to operate the gene bank will vary with the size of the repository. Common to all sizes, however, is the need for three different disciplines: genetics,

reproductive physiology and cryobiology, and information systems and database development. Minimally, the gene bank requires

1) a curator (whom will likely have a genetics background),
2) technical support to cryopreserve germplasm, and
3) technical support to develop a database and input information into the database about the samples in the collection.

The curator has to develop targeted collection goals for each species and breed within species of interest. Obviously, this task is a significant responsibility for one person and therefore, in formulating collection development plans the curator may need to seek input from a wide array of livestock expertise in the country. Such solicitations should address not only to the research community but also the livestock industry, including groups involved in raising various livestock breeds.

As gene bank activities increase in size and scope there will be need for increased staffing. Much of this increase is dependent upon the flow of germplasm into the repository. But additional technical needs arise with increased size and the need to employ a broader range of reproductive and cryopreservation technologies on different species, and the need to quantify and understand the genetic diversity which may include the utilization of molecular genetic techniques. With additional requirements comes the need for additional scientific and support. Of course many of these additions can be addressed by integrating conservation activities with pre-existing laboratories that have the additional expertise (e.g. laboratories that specialize in molecular genetics). Nevertheless, the gene bank absolutely needs to have a reasonable level of competency to execute its conservation mission.

A critical factor for smooth operation of gene banks is proper training. Regardless of his or her particular role, all gene bank employees should have an initial course of specific orientation and training when beginning employment. Furthermore, training must be continual, and especially rigorous in biosecurity.

5.7. Continuity of operations

As with any important national resource, gene banks need to have in place procedures for handling the collection in the event of an emergency. Such emergencies might include the loss of electricity, flooding, earthquakes, civil unrest, or a disease outbreak that impacts either the animal or human population. If such an event were to take place, the predetermined plan would provide a set of guiding actions with minimal impact on the safe maintenance of the collection.

The following considerations must be made to ensure sustainability of the gene bank and to guard against loss of the stored materials in the case of a natural or man-made disaster – some of these considerations were already mentioned with regard to gene bank security, but their importance cannot be overstated:

- Establishing the gene bank in a location where hazards such as earthquakes, floods and tornados are minimized.
- Split the collection into two parts and store in geographically different facilities. This type of redundancy provides potential protection against earthquakes, floods, or tornados. This decision will depend on the finances available and the level of security afforded by the primary bank. In general, only the facilities for long-term storage (Core and Historical Collections) will need to be replicated, so the costs for two gene banks will be much less than double the cost of a single bank.
- Develop contingencies for moving the entire collection or a pre-determined subset of the collection in the event of some type of civil unrest is an option for some gene bank managers to explore.
- Assigning minimal numbers of staff required to report to the gene bank in the event of any of extraordinary events.

- Develop plans to acquire supplies from alternative sources (i.e., LN2).

Once contingency plans have been developed they need periodic review to ensure the developed options are still viable.

6. DEVELOPING GENE BANK COLLECTIONS

Developing and updating gene bank collections is a long-term endeavour involving several processes. Major steps include: understanding the dynamics of the population of AnGR of interest, determining the status of the population (e.g., whether numbers have dropped to a predetermined critically low level), establishment of collection targets for germplasm (semen, embryos, or oocytes) or tissue, and the selection of animals from which samples will be obtained for the collection.

As mentioned in Chapter 1, the determination of which breeds and animals to have represented in the gene bank is dependent upon a country's National Strategy and Action Plan (NSAP) for AnGR and the country's capacity to obtain and store the samples. For countries lacking gene banks, the decision on needs for conservation of each AnGR should be undertaken as a first step to determine if gene bank is the best strategy for conservation. Factors to consider with regard to the adoption of cryoconservation include both the accessibility of the animals for collection and the technical and financial capacities within the country for processing and storing the samples when collected.

A primary consideration for all gene banks has to be the acquisition of sufficient quantities of germplasm for the reconstitution of populations and that the samples collected sufficiently represent the genetic diversity of the population in question.

6.1. Choice of populations to include in the gene bank

Before initiating collection activities gene bank managers will have to assess various breeds and species in the country for the purpose of determining where to start collection development. More details on the identification of breeds at risk and prioritization of AnGR for conservation are presented in the *FAO Guidelines for In Vivo Conservation of Animal Genetic resources.* Such decisions can be made on the basis of a breed's population, potential genetic uniqueness, economic importance, and cultural importance. In general, the choice of breeds for conservation should be a group decision, undertaken by the National Advisory Committee for AnGR[11] or another committee of expert stakeholders. Nevertheless, gene bank managers and curators should have the flexibility to be pre-emptive and be allowed to initiate collection of germplasm on breeds that are viewed to be at an extreme state of endangerment or critical to the country's livestock sector.

Various quantitative measures can be used to better clarify or prioritize breed collections. In general, there are two primary factors that determine the priority for conservation of a breed,

1) level of endangerment or risk of extinction, and
2) conservation value.

6.1.1. Level of endangerment

From a quantitative perspective, the level of endangerment can be thought of as expected future population size. The best measurable indicator of future population size is the current population size. Measurements of past population size in the past can improve the prediction of future population size, as trends can be observed and extrapolated into the future. Unfortunately, breed level population data are often not available, especially in situations where breed associations are not established. Although many countries undertake periodic censuses of livestock, nearly all countries do their censuses counting only the animals within a species, without specifying the

[11] See *FAO Guidelines on Preparation of national strategies and action plans for animal genetic resources* ftp://ftp.fao.org/docrep/fao/012/i0770e/i0770e.pdf.

breeds they belong to. As a result it may be relatively easy to obtain a picture for an industry but gaining clarity about breeds within a species is more difficult. Introducing a routine national census for monitoring of breed populations should be part of a country's NSAP as well as a standard procedure in reporting on implementation of the *Global Plan of Action*. FAO has produced *Guidelines on Surveying and Monitoring of Animal Genetic Resources* to assist countries in this endeavour. If a census is to be performed it can be performed in two stages: (i) a partial census to identify breeds that may be at risk; (ii) a complete census (or as near complete as is feasible) of those breeds identified in the partial census as possibly being at risk.

If no national census exists, gene bank managers (or national working groups on AnGR conservation) may wish to initiate their own census collecting protocols, provided the necessary financial and technical support is available. The previously mentioned *Guidelines on Surveying and Monitoring of Animal Genetic Resources* can be consulted during this procedure. If new initiatives are to be employed to develop census or a census based upon a partial sampling of information, it is critical that the sampling procedure be well designed and it is recommended that gene bank managers enlist the support of statisticians to design an appropriate sampling strategy. When breed associations are present, collaboration is recommended as these organizations typically keep track of annual registrations and may be willing to share such information.

Factors beyond population size can also influence level of endangerment. Breeds that are distributed across more farms and/or a wider geographic area tend to be at less risk than breeds with a more concentrated distribution. When more farmers possess a breed, then the impact on the population size of an action by any single breeder will be less. Wider geographical distribution decreases the risk that the entire population can be wiped out by a single geographically-concentrated catastrophic event, such as a disease outbreak. Increased crossbreeding and increased inbreeding are also activities that put an AnGR at risk. The number of animals may stay the same, but the amount and diversity of the original AnGR is decreased.

6.1.2. Conservation value

As noted in Chapter 1, conservation of AnGR may be undertaken to meet various objectives. These objectives and their relative importance are likely to vary from country to country. In turn, variability will exist among breeds in their relative contribution to meeting these conservation objectives. The following factors will influence the conservation value of a given breed (Ruane, 2000):

- **Genetic uniqueness of a breed** – genetic distinctiveness is an important criterion for establishing conservation priorities. Understanding the genetic history of a particular breed or formally estimating genetic distance among breeds will assist in determining breed uniqueness.
- **Genetic variation within a breed** – genetic variation provides a given AnGR the capacity to adapt and allows for genetic response to selection. Conserving the most genetically diverse breeds is the most efficient way to conserve the diversity of a given species.
- **Traits of economic importance** – breeds that are genetically superior for economically important traits (at present or foreseen in the future) should receive priority in conservation. This decision requires evaluation of both current and potential importance of particular breed characteristics and performance.
- **Unique traits** – breeds with special behavioural, physiological or morphological traits should be given high consideration for conservation.
- **Adaptation to a specific environment** – the adaptation of breeds to specific environments is likely to be under some genetic control. Thus conservation of these AnGR may be important and thus should increase the conservation value. Breeds that perform important environmental services are often important to conserve.
- **Cultural or historical value** – breeds with special cultural or historic values merit consideration for conservation.

- **Species a breed belongs to** – there is a suggestion that while the above criteria are important in selecting breeds within species, some consideration should also be given to the species to which the breeds belong, to ensure that some financial resources are allocated to all important livestock species and to account for the costs and likelihood of success of a conservation programme.

Accounting for all of these factors can be quite difficult. Clearly, prioritization of breeds for collection and entry into the gene bank can be performed in a number of ways depending on national need. Advisory committees of experts and including stakeholders from industry and breeders' associations can advise the gene bank about the endangerment level of various populations and their genetic, economic and cultural importance.

Molecular markers may be used for evaluating genetic distances and diversity of breeds (See *FAO Guidelines on Molecular Genetic Characterization of Animal Genetic resources*). Various objective methods have been proposed for incorporating molecular measures of diversity in conservation decisions (See review by Boettcher *et al.,* 2010). These methods typically allow for the simultaneous consideration of level of endangerment and the genetic and non-genetic factors listed above. Their use is discussed in more detail in the *FAO Guidelines for In Vivo Conservation of Animal Genetic resources*, because prioritization should be done as part of an overall general conservation plan, not only for cryoconservation.

Although formal prioritization methods may increase the efficiency of conservation decision making, a willingness to be flexible in establishing collections so as not to miss unexpected needs and opportunities for collection when they arise will substantially enhance collection development. When particular AnGR are at high risk of extinction or if collection of a given AnGR can be done at a very low cost, their collection can be justified irrespective of the availability or result of formal prioritization analyses.

6.2. Collection targets for reconstituting populations

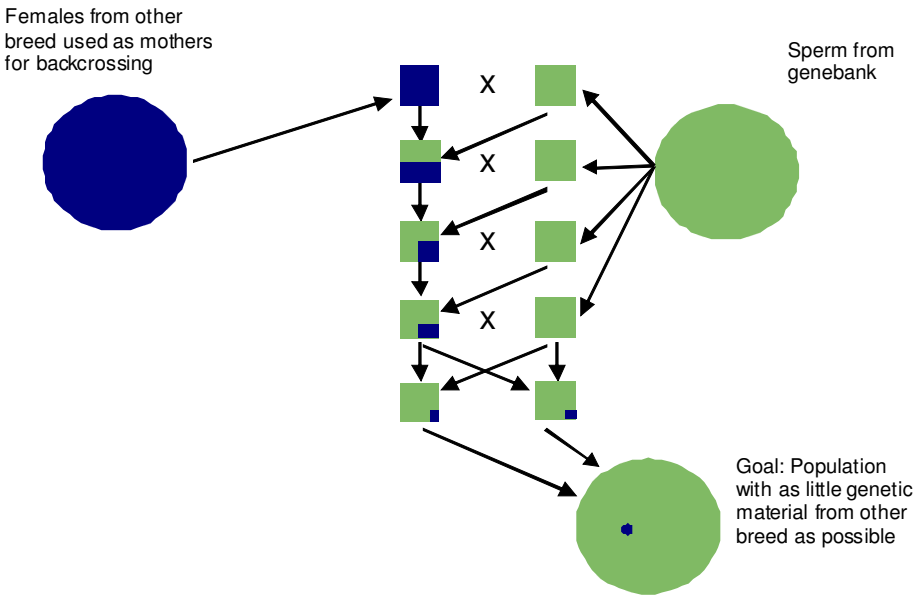
Once the decision has been taken on the particular breeds and populations to be collected for the gene bank and the type of germplasm to be collected the next step is to determine the types and amount of germplasm to be collected. These quantities will vary depending on the conservation goal, germplasm and species. In general, reconstitution of extinct populations will usually require the greatest quantity of germplasm.

6.2.1. Targets for cryopreserved semen

When semen is cryopreserved, the principal method for reconstituting a breed or population is through backcrossing (Figure 6.1). One starts with females of a common breed and with four backcross generations it is possible to reconstitute over 90% (4 generations ~ 94%; 5 generations = ~97%)[12] of the conserved breed's or population's nuclear genome. The subsequent sections illustrate how semen can be used to reconstitute a population and the amount of germplasm needed to accomplish this goal.

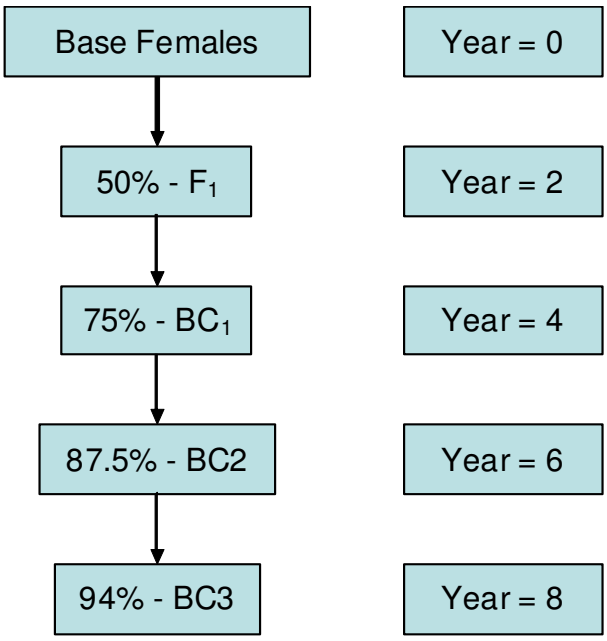
[12] Proportion of target breed recovered = $1 - .5^b$, where b is the number of backcross generations.

Figure 6.1. Demonstration of population reconstitution with cryoconserved semen.



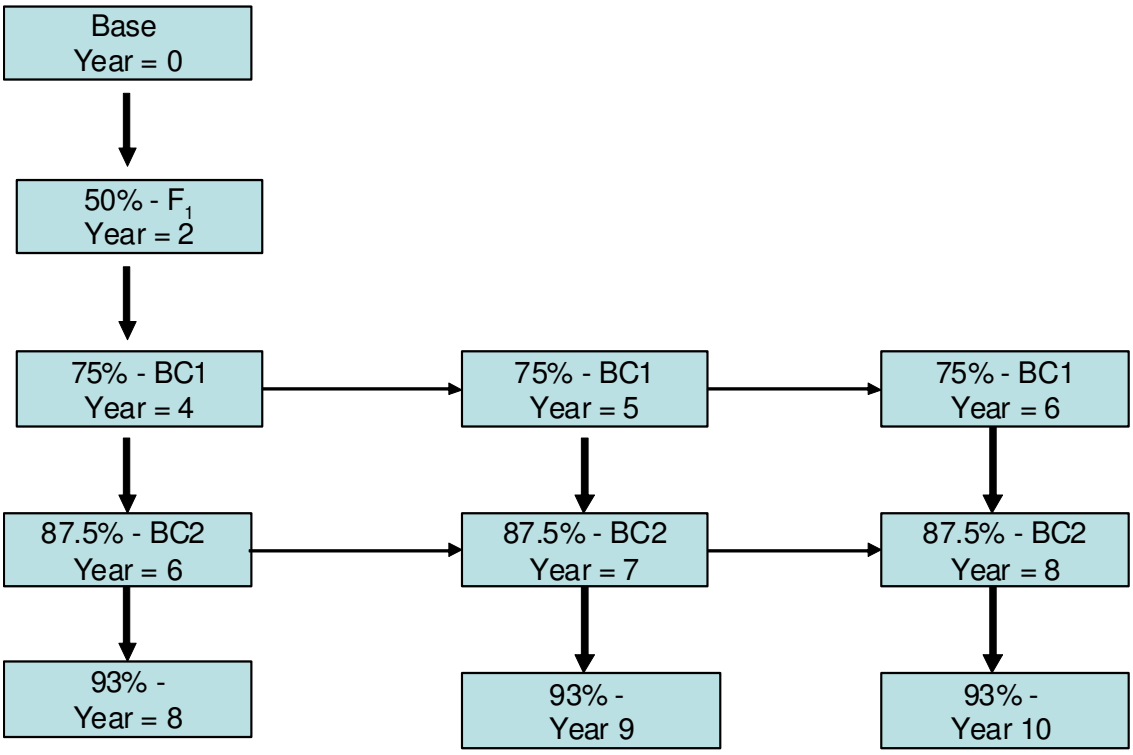
The length of reproductive cycles and conception rates impact the speed and efficiency with which populations can be reconstituted. To better establish collection goals, a view on how the cryopreserved material will be used to reconstitute a breed needs to be quantified. Previous reports (FAO, 1998; Boettcher *et al.,* 2005) used a single backcrossing approach, such as shown in Figure 6.2. In this approach, females are used as mothers only a single time in their lives. This approach allows reconstruction with maximum speed and simplicity. All animals of the same age will be of the same generation and will have the same proportion of the reconstituted breed. Because females are used only once, the quantities of semen needed for reconstituting a population are quite large. These large projected quantities are primarily due to the 50:50 sex ratio that must be assumed, which in the early generations result in a large number of excess males.

Figure 6.2. Standard backcrossing plan for breed reconstitution (using cattle as an example)



Several livestock species, including cattle, buffalo, small ruminants and horses have longer reproductive cycles and/or smaller family sizes than do other livestock species such as pigs and poultry. Therefore, advance strategic planning with regard to mating has the potential to drastically improve the efficiency of gene banking and breed reconstitution. The mating plan in Figure 6.3 uses a slightly different backcross scheme. This approach allows females in the first, second and third backcross generations to be mated up to three times. This strategy allows these animals to have a much greater chance to produce a female offspring. Expanding the mating opportunities of these later generations results in smaller amounts of semen use, and decreases the cost of maintaining the animals used in reconstituting the breed of interest. The principal drawback to this approach is that it will take a slightly longer time for reconstituting the population (e.g., 1 to 3 years).

Figure 6.3. Alternative efficient mating plan for breed reconstitution.



6.2.1.1. Ruminants and horses

Using the breeding plan described above, quantities of semen needed for reconstitution of a breed were calculated for a 150% breed replacement plan. These results are presented in Table 6.1. The "150% breed replacement plan" means that the gene bank will save 1.5 times the germplasm expected to be necessary to reconstitute a breed (i.e., to obtain 25 males and 25 females with 94% of the conserved breed). Planning at the 150% level provides flexibility and additional amounts of germplasm that may be needed to compensate for lower than expected pregnancy or survival, excess animals of one sex, or other failures in the reconstitution process. Targeted quantities of semen are provided for varying rates of pregnancy, realizing that under different circumstances conception rates do vary. Also used as a variable in Table 6.1 is the number of founder breed females used to start the reconstitution process. Critical to the reconstitution process is the final number of animals that will be generated with 93.75% of the targeted genome are also provided (in parenthesis) in Table 6.1. (Note that the reconstituted population sizes are also estimates of N_e.) For a number of conception rates and initial number of animals bred, the N_e is below 50, the

recognized target. However, this deficiency can be managed by having extra males with semen in the repository which can be used to further broaden the genetic base of the newly regenerated breed.

Table 6.1. Doses of semen and resulting effective population size given varying pregnancy rates and number of animals used to reconstitute a breed of cattle, small ruminants or horses at the 150% level.

Founder females (N)	Pregnancy Rate			
	0.4	0.5	0.6	0.7
75	...[1]	...	449 (26)	460 (37)
100	...	564 (22)	599 (35)	615 (49)
150	771 (17)	846 (33)	897 (53)	...
200	1 029 (23)	1 128 (44)	...	...
250	1 287 (29)	1 410 (55)	...	...
300	1 544 (34)	...	...	...
350	1 800 (40)	...	...	...
400	2 058 (46)	...	...	...

[1] Missing values indicate that results are not practical, resulting in either too few (upper left corner) or too many (lower right) animals or requiring large quantities of semen.

Given that target breed reserves are established, estimates of the quantities of semen per male are presented in Table 6.2. For many mammalian species, the quantities shown in Table 6.2 are achievable in a single collection. Nevertheless, gene bank managers should still follow the conservative approach of storing samples from at least two collections from the same male separated by at least 2 weeks.

Table 6.2. Number of doses per male needed for reconstitution given pregnancy rates and number of founder females used to reconstitute a breed at the 150% level when semen from 25, 50 or 100 males are in the repository.

Founder females (N)	Pregnancy rate											
	0.4			0.5			0.6			0.7		
	Males (N)			Males (N)			Males (N)			Males (N)		
	25	50	100	25	50	100	25	50	100	25	50	100
75	...	...	...	...	...	...	18	9	5	19	10	5
100	...	...	...	23	12	6	24	12	6	25	13	7
150	31	16	8	34	17	9	36	18	9	...	...	...
200	42	21	11	46	23	12	...	...	...	...	...	...
250	52	26	13	57	29	15	...	...	...	...	...	...
300	62	31	16	...	...	...	...	...	...	...	...	...
350	72	36	18	...	...	...	...	...	...	...	...	...
400	83	42	21	...	...	...	...	...	...	...	...	...

With cattle, and potentially other species in the future, the utilization of sexed semen would dramatically change the projected amount of semen needed to reconstitute populations. The results in Tables 6.1 and 6.2 assumed a sex ratio of 50:50 for males:females. With sexed semen the number of doses could be decreased by 30 to 45%.

Another option that countries may consider to decrease the number of doses of semen (and time for reconstitution) is to accept a larger proportion of the founder breed in the final “reconstituted” population. For example, if a ratio of 7-to-1 (87.5%) of the reconstituted and founder breeds is acceptable, the number of backcross generations can be decreased to three and the amount of

semen can be decreased by 30% or more. This option may be particularly attractive in situations where reaching the goals in Table 6.1 would be difficult for practical reasons.

6.2.1.2 Swine

The relatively high reproductive capacity and shorter gestation length of pigs enables a quicker breed reconstitution, when compared to cattle and horses. Significant numbers of animals are not needed to start the reconstitution process due to the prolificacy of the species. To a certain degree, the innate reproductive ability of the sow compensates for the occasionally high mortality of sperm cells during the cooling and cryopreservation process. In Table 6.3, the numbers of doses of semen needed for reconstitution are given as well as the numbers of offspring reconstituted at the 93.75% level. The data are based on the assumption that 6 piglets from each litter reach breeding age. This is a very conservative estimate given the results of Spencer *et al.,* (2010), who obtained a 74% pregnancy rate and an average litter size of 11 when females were bred through AI. Quantities of semen needed per male are presented in Table 6.4. As with the other species (Table 6.2), these quantities can potentially be obtained from a single collection, but a more conservative practice is to obtain two collections from each boar.

Table 6.3. Number of doses of semen1 and resulting effective population size given varying pregnancy rates and number of animals used to reconstitute a breed of swine at the 150% level.

Founder females (N)	Pregnancy rate 0.4	0.5	0.6
15	2 880 (56)	2 760 (66)	2 520 (72)
25	4 800 (96)	4 560 (108)	4 200 (126)
50	9 600 (192)	9 000 (225)	8 400 (252)

[1]Assumes 20 0.5 ml straws per insemination, with a total of one billion cells per insemination.

Table 6.4. Number of straws per boar needed for reconstitution given pregnancy rates and number of animals used to reconstitute a breed at the 150% level, when semen from 25, 50 or 100 males are in the repository.

Founder females (N)	Pregnancy rate 0.4 Donor males (N) 25	50	100	0.5 Donor males (N) 25	50	100	0.6 Donor males (N) 25	50	100
15	116	58	29	111	56	28	101	51	26
25	192	96	48	183	92	46	168	84	42
50	384	192	96	360	180	90	336	168	84

6.2.1.3. Chickens

With chickens, the breed categorization used with mammalian species may not be the primary conservation emphasis. Although breeds are important, industrial populations comprised of distinct lines may be of greater importance, depending on the country. In addition, poultry breeders and scientists have created numerous research populations, many of which can be categorized by Mendelian traits controlled by single genes or quantitative trait.

A further consideration for chicken population reconstitution is that cryopreservation has been problematic in recent years because the contraceptive effects of glycerol on hen fertility. This effect is discussed under the cryopreservation protocols. Among the solutions for this problem are to use alternative media and to perform intramagnal instead of intravaginal insemination. By using the intramagnal approach, the number of units of semen needed to reconstitute a population can be significantly reduced (Blackburn *et al.,* 2009).

Table 6.5 outlines the resources needed to create gene banks for chickens, according to the objective for the eventual use of the stored materials. These numbers were obtained based on a number of assumptions regarding efficiency of reproduction and survival of resulting offspring. Specifically, the assumptions used in the calculations for Table 6.5 include:

- Two fertile eggs produced per hen per insemination,
- 1.4 fertile eggs hatched per hen per insemination,
- Of hatched chicks, 85% become adults,
- Two inseminations per 0.5 ml straw, and
- Sex ratio of surviving chicks of 50:50.

Table 6.5. Reconstitution approaches for varying chicken populations.

Item	Single gene introgression	Quantitative trait lines (5 generation backcross)	Breed (5 generation backcross)
Total straws used[a]	7	127	257
Initial number of hens	14	100	140
Inseminations for entire reconstitution process	14	254	513
Generation number for multiple intramagnal inseminations per hen (N/hen)[b]	0 (0)	4 (3)	3 (3)
Final number of target population produced (generation number)	16 (1)	44 (5)	62 (5)
Minimum number of straws for 150% reconstitution	11[c]	191[c]	386[c]

[a] Based upon a motile sperm concentration of 200 X 10^6 (Purdy *et al.,* 2009).

[b] Generation 3 and 4 hens will have 87.5% and 93.7% of the genome of interest.

[c] Assumes a 0.5 ml straw and two inseminations per straw.

6.2.2. Embryo use in breed reconstitution

As noted in Chapter 4, embryos have some advantages and disadvantages relative to semen with regard to reconstitution of a population. Their principal advantage is the speed with which breeds can be reconstructed (less than 5 years). In addition, the use of embryos ensures the conservation of a breed's entire genome, whereas the mitochondrial genome is lost when only semen is used and a certain proportion of the founder breed for backcrossing will be present. Embryos could also be particularly important for breeds with extremely unique characteristics that would be very difficult to re-establish using semen in a backcrossing scheme. The Angora goat is an example of this type of situation. To re-establish the fibre quality from crossing with a founder population of non-fibre producing goats would be very problematic, from an economic standpoint, as the fibre from initial backcross generations would be of very little economic value.

However, embryos are significantly more expensive to collect and require greater technical capacity than semen (Gandini *et al.,* 2007) and ET is not possible for all species of livestock. Biologically, the embryo offers the complete genetic complement of the breed, but the genetic combinations formed when making the embryos can become dated and therefore their utility after long-term cryostorage is an issue of which gene bank managers should be cognizant.

Table 6.6 shows the number of embryos required to be cryopreserved in order to obtain a reconstructed population of 25 breeding males and 25 females, depending on the survival of the embryo from thawing to birth and the subsequent survival from birth to breeding age. Ideally, the numbers of embryos per donor will be nearly evenly distributed across at least 25 donor females, each mated to a different male (or multiple males), to capture the maximum amount of diversity possible from the existing population.

Table 6.6. Number[a] of cryopreserved embryos required to reconstitute a breed population consisting of 25 males and 25 females to a 150% level, as a function of survival from transfer to birth and from birth to breeding age.

Embryo survival, thawing to birth	Offspring survival, birth to breeding age			
	0.6	0.7	0.8	0.9
0.2	625	536	469	417
0.3	417	358	313	278
0.4	313	268	235	209
0.5	250	215	188	167
0.6	209	179	157	139
0.7	179	154	134	120

[a] Numbers of embryos (n_{emb}) were obtained according to the formula $n_{emb} = 1.50 \times 25 (0.5 \times s_e \times s_o)$, where 1.50 is a multiplier to obtain the 150% level, 0.5 is the sex ratio and s_e and s_o are survival rates to birth and breeding age, respectively (Gandini and Oldenbroek, 2007).

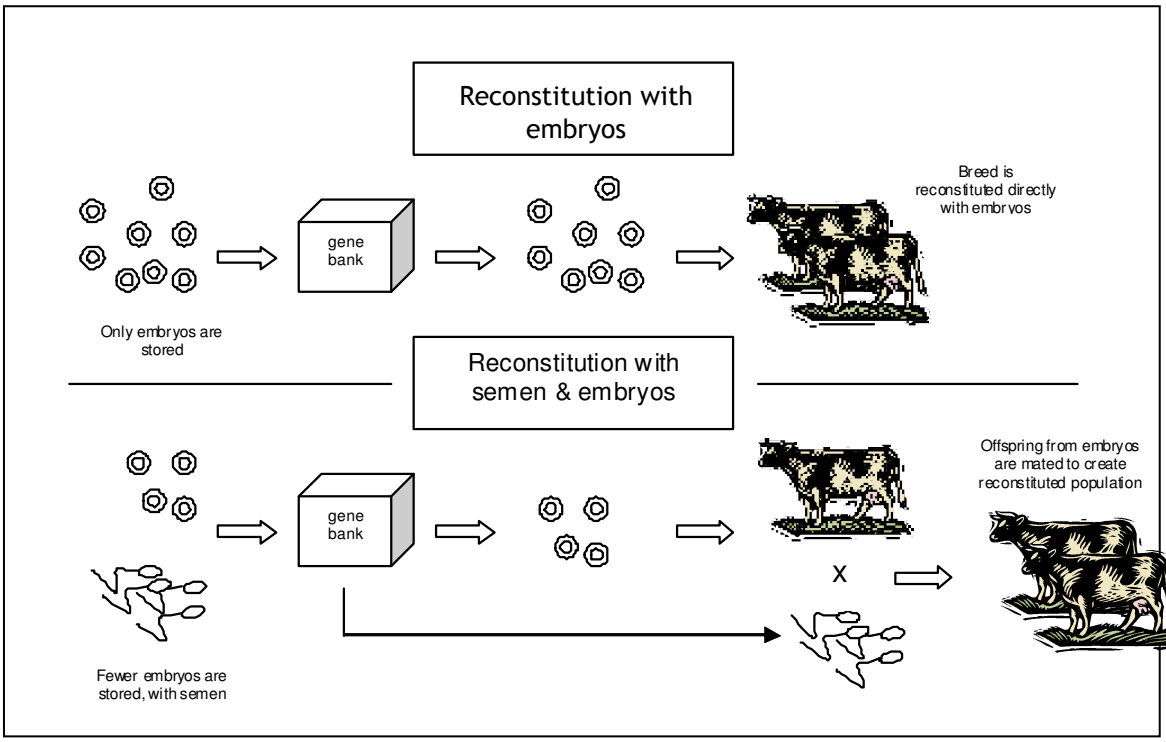
In cases where sampling of 25 donors is not possible (or simply to economize), another option that may be considered is a hybrid scheme in which one stores a combination of semen and embryos (Boettcher *et al.,* 2005). In this strategy, smaller numbers of embryos are required than listed in Table 6.5. Embryos are used to produce a population initially consisting of fewer than 25 breeding males and 25 breeding females, but these animals are subsequently mated with each other and with the stored semen to eventually achieve a population of the desired size after one or more years (Figure 6.4). This approach can reduce costs relative to storing only embryos, while still allowing the maintenance of the mitochondrial genome and usually decreasing the time required for breed reconstitution relative to the backcrossing scheme utilized when storing only semen (i.e. Figures 6.1 to 6.3).

This approach requires some compromises, however. Although reconstitution will usually be faster than with stored semen, multiple breeding cycles are nevertheless required to reach the final goal of 25 animals of each sex, whereas this goal can be achieved in a single breeding cycle with embryos. Furthermore, genetic relationships in the reconstructed population increase (and N_e decreases) as the number of embryos decreases, as does the probability of failure to reconstitute the new population. Therefore, a hybrid scheme with fewer than 30% the embryos required for an embryo-only reconstitution plan (i.e. Table 6.6) is not recommended. To increase N_e, the sires with semen in the bank should be different from the sires of the stored embryos.

A conservative approach to germplasm collection development would be to acquire semen samples as previously proposed (i.e. in section 6.2.1) and, where possible, acquire embryos opportunistically to enhance the quality of the banked material. Collecting and maintaining sufficient embryos in the gene bank to generate 10 to 20 females would speed reconstitution and it would insure that mitochondrial DNA was captured in the collection (Boettcher *et al.,* 2005).

Cryopreservation of embryos is most effective in the ruminant species. With swine, embryo cryopreservation can be achieved but the success rates are lower. Of course in poultry embryo preservation is not possible and therefore to capture the genetic complement of a population primordial germ cells (PGC) have to be harvested and cryopreserved (Section 4.8.3.). Moore *et al.,* (2006) demonstrated that PGCs can be effectively cryopreserved. But successfully inserting those cells into recipient eggs is problematic with about a 4-8% success rate (Petitte *et al.,* 1990).

Figure 6.4. Reconstitution using only embryos versus a reduced number of embryos and semen.



An important aspect of controlling the genetic quality of the sample of embryos is undertaken during the period of collection, by obtaining the required number of samples to achieve the preset objectives. Before genetic reserves are established in the form of embryo banks, it is necessary to consider the number of animals available and to decide whether their genetic potential will allow collection of a sufficient number of unrelated embryos for subsequent reconstitution of a breed. Ideally, more than 25 donor females would be preferable, to increase the genetic variability in the reconstituted population. The final decision will depend on availability of donor females and costs of the system implemented for access to germplasm. For example, if donor females must be purchased by the gene bank from the breeders, the acquisition of exactly 25 donors may be financially optimal. However, if payment is made to the breeders for each collection (or if no payment for access is necessary), then the sampling of more than 25 donors may have costs similar to the sampling of exactly 25 donors. For rare breeds, gaining access to 25 donor females may be problematic, however.

Multiple embryo recoveries may be needed from each superovulated donor female. Embryo recovery following superovulation is notoriously variable, especially in cattle. The numbers of embryos per collection for a well-managed donor may range from 0 to 40, with an average of 5 or 6 transferable embryos. In addition, females that do not respond well to superovulation on the first collection have a tendency not to respond well in subsequent attempts.

Therefore it is important that the males used to produce the matings are not associated with the same female throughout the collection period, otherwise one or a few sires may dominate others in the re-established breed. Furthermore, even though no overt selection is taking place, natural selection may be acting and there is an implicit penalty arising from full sibs: deleterious genes passed on by an individual of one sex penalizes not only its own future contribution to the population but also that of its healthy mate. Avoiding full-sib donors to obtain offspring numbers helps spread the risk (Woolliams, 1989).

6.2.3. Breed reconstitution with oocytes and semen

When oocytes and semen are stored in the gene bank, the number of oocytes required can be determined by using a slight modification of the formula applied to compile the data in Table 6.6.

$$n_{ooc} = 1.50 \times 25\ (0.5 \times s_e \times s_o \times s_{ivf}),$$

where, n_{occ} is the number of oocytes to be banked, 1.50 is a multiplier to obtain the 150% level, 0.5 is the sex ratio and s_e and s_o are survival rates to birth and breeding age and s_{ivf} is the success rate of the IVF procedure used to obtain an embryo from oocyte.

The number of doses of semen stored should be sufficient to fertilize all of the stored oocytes. A conservatively high estimate is one dose of semen per oocyte stored, but a single dose can fertilize multiple oocytes. Ideally, the number of males from which semen is stored should be at least as large as the number of female donors of oocytes.

6.3. Utilization of gene bank material in live conservation and breeding

As noted previously (Chapter 3), in addition to the reconstitution of a breed after its extinction, there are several other opportunities to use gene bank material, both in the near and long term.

First of all, the use of gene bank material for breeding animals in live populations can be helpful to control inbreeding rates or to revitalize populations. Controlling the rate of inbreeding in a population is important to maintain genetic variation. The N_e of a population should be at least 50, which corresponds to a rate of inbreeding of 1% per generation. Gene bank collections can play an important role in maintaining genetic variation of a breed and will in fact increase the N_e of a breed or population. (See Box 6.1 for an example of how this approach is currently being applied in the Netherlands.) Obviously, the males whose semen is to be used from the bank must not be part of the live breeding population. When managing a small population, three to ten males should be identified each year and their semen should be stored for future use. At least 20 and 100 doses of semen from each male should be conserved, depending on the reproductive capacity of the species (low capacity → more doses) and population size (larger population → more doses). Because this practice will involve the use of "old" germplasm, it limits the amount of genetic progress that can be made for a given trait. However, the main objective is to maintain a high level of genetic diversity in the population.

Box 6.1. Use of gene bank semen for revival and support of the breeding programme of the endangered Dutch Friesian Red and White cattle breed

In 1800, the cattle population in the province of Friesland in the Netherlands consisted mainly of Red Pied cattle. During the past century, (export driven) preference for the Black and White phenotype, followed by sustained import and crossing with Holstein Friesians, resulted in a sharp decline of the Red Pied population, so that only 21 Red and White individuals (4 males and 17 females) were remaining in 1993. At that point in time a group of owners started the Foundation for native Red and White Friesian cattle. In collaboration with the newly created gene bank for farm animals, a breeding programme was developed. Semen from sires preserved in the gene bank in the 1970s and 1980s was used to breed females. Male progeny were raised by breeders, who were granted a subsidy from the gene bank. Semen from these males was collected, frozen and later used under new contracts. Since then, the breed increased in number, reaching 256 registered living females and 12 living males in 2004. In addition to the living populations, more than 10 000 doses of semen of 45 bulls are stored in the gene bank and kept available for AI. The combination of the living population and the gene bank stock results in a much larger N_e than is represented by the living population only.

Secondly, the material can be used in a cross-breeding system for introgression of specific characteristics into live populations. Breeders may want to introgress desirable characteristics of a cryopreserved breed into an existing breed. Introgression can be based on phenotypic information, and the desirable characteristics can be maintained in the next generations by continuous selection. Alternatively, the genes underlying the desirable characteristics can be identified and molecular markers can be used to maintain the desirable genomic regions or traits. Introgression or crossing cryopreserved populations with live populations may also result in completely new breeds. Introgression generally involves only a single cycle of breeding to the conserved breed that provides the desired gene. In subsequent generations, the live animals are bred *inter se* or

backcrossed to purebred animals of the live population. Therefore, the number of doses stored will depend on the number of females in the live population that will be subject to crossing to initiate the introgression process. Box 6.2 describes the use of banked germplasm to introgress genes into a herd of swine used for research.

Box 6.2. Reconstituting a Research Pig Line

Gene banks have an important role in backing up research populations. Purdue University in the United States had developed a line of pigs that were either homozygous or heterozygous for both the Napole and Halothane genes, which negatively impact pork quality in animals with the homozygous recessive genotype. In 2003, Purdue decided to discontinue this population and chose to have samples of semen from three carrier boars frozen and banked by the National Animal Germplasm Programme (NAGP). In August 2007 the University decided to reestablish a population where the recessive homozygous condition was present so that it could be used to research meat quality. Samples of the semen stored with the NAGP were therefore transferred back to Purdue and sows were inseminated. The breeding results were a 100% pregnancy rate and an average litter size of 7.7 pigs. Genotyping was performed on the resulting boars and 14 of 25 were heterozygous for both genes. With the F_2 population formed, several boars were homozygous for both mutant genes. This activity represented the first time that a livestock research line was cryopreserved, discontinued, and re-established using germplasm frozen and stored by a gene bank.

For any breeding program, regardless of population size storage of periodic cryogenic storage of genetic material is recommended to serve as a back-up in case (genetic) problems occur. Especially for populations with a low N_e, a cryo-aided live scheme can be very beneficial, mainly because it will result in prolonged generation intervals and therefore a larger N_e. Intensely selected breeds can actually have a small N_e, even if the actual number of animals is very large (Bovine HapMap Consortium, 2009). It is important to collect new genetic material regularly in order to maximize genetic diversity or to keep a representative back-up of an existing population.

6.4. Selection of individuals for cryopresevation

Section 3.2 of these Guidelines established the first target for germplasm collections – that a breed collection should be able to reconstitute a population with an N_e of 50 animals. However, for the gene bank manager the issue quickly turns to which animals within a breed should be placed in the repository for any of the collection categories. Genetics are often among the primary considerations, but reproductive and sanitary aspects must also be accounted for.

6.4.1. Genetic aspects

Here the major consideration is to select animals that have the least genetic relationships to one another. Several avenues exist depending upon existing information and resources available to make choices.

- When pedigree information is available, simple procedures can be applied to ensure that animals are not closely related, such as avoiding the selection of animals with common grandparents.
- More formal analyses can also be undertaken, such as the application of genetic contribution theory to select the least-related group of germplasm donors (Meuwissen, 2002). Clustering approaches can be employed to group animals that are closely related and identify clusters that are genetically distant from one another (See Box 6.3).
- Donors should be chosen from within lines if line breeding is practiced.
- With or without pedigree information, the opportunity exists to use various molecular DNA approaches to determine the genetic uniqueness of various animals or subpopulations within a breed. A principal obstacle to this approach is that a wide sampling of the animals within the breed needs to be performed and many more animals will need to be genotyped than are actually needed for the gene bank. Alternatively, gene bank managers may consider to collect germplasm samples concurrent to taking blood or

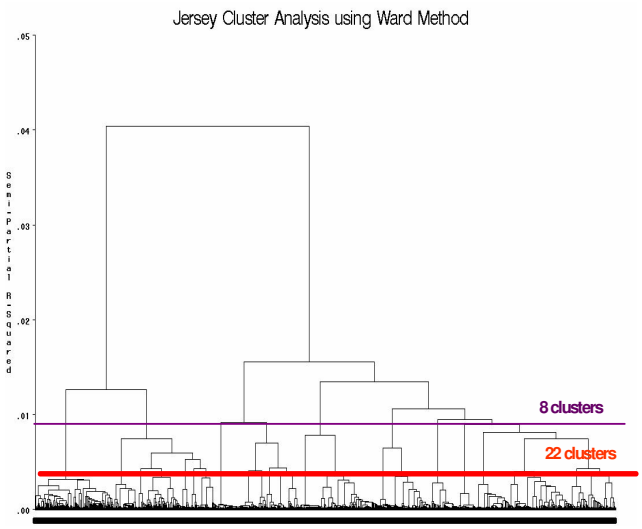
tissue samples, and to use the resulting genotypic data to assist in the utilization of the stored material to decrease genetic relationships in the reconstituted population.

- Genetic markers can also be used to identify introgression from other breeds, the level of which may differ among sub-populations or areas. This type of information is useful in determining how genetically pure are the targeted animals.
- If there is no reliable animal registration available and resources are insufficient for use of molecular genetics, donors should be carefully identified according to geography, phenotype and herd history.
- Particularly when pedigree information is not available, donor animals should be chosen from different areas and herds, considering genetic flows (i.e. exchange of animals) among herds and areas. For example, animal choice can be made by collecting along line transect(s) drawn through maps of the regions of the country where the breed is located. Adequate geographic spacing should help insure that the level of genetic relationships among collected animals is low.
- Even when animals are taken from geographically distant locations, owners should be interviewed to determine how unrelated their animals are from the immediate surrounding population and from other more distant flocks or herds.

Box 6.3. Selection of bulls for the National Animal Germplasm Programme in the United States of America

Cluster analysis was use to evaluate the bulls of the Jersey breed stored in the United States gene bank (Blackburn, 2009). Pedigrees of the bulls in the gene bank (n = 537) were evaluated along with pedigrees of the most popular bulls in 2004 and 2005. Genetic relationships among the bulls were used to assign the bulls to clusters, which were visualized in a dendrogram or "tree" diagram (see the figure below). The figure demonstrates how one can define any given number of clusters (e.g. 8 or 22 clusters) by drawing a horizontal line across the dendrogram. The number of bulls in each cluster, the mean genetic relationship per cluster, and the number of bulls in the gene bank from each cluster were calculated for 22 clusters (See the table below). Several clusters were poorly represented in the repository (e.g. clusters 4, 15, 16, and 21) and efforts were therefore made to acquire samples to fill those gaps in the collection. A similar procedure could be followed to initially select animals for a gene bank, by choosing similar numbers of animals from each cluster.

Cluster	n	Mean Rel.	No. in Rep.
1	350	.18	85
2	98	.20	30
3	451	.05	105
4	50	.23	2
5	115	.35	8
6	214	.21	38
7	193	.25	16
8	198	.11	13
9	342	.21	42
10	161	.20	37
11	116	.36	17
12	126	.22	36
13	142	.19	7
14	116	.33	15
15	62	.26	
16	104	.20	2
17	156	.26	35
18	105	.33	22
19	70	.30	18
20	86	.28	14
21	49	.22	2
22	46	.22	10



n = number of bulls per cluster
Mean Rel. = mean additive genetic relationship within the cluster
No. in Rep. = number of bulls selected for the gene bank from each cluster

6.4.2. Reproductive aspects

Only a small sample of animals can be represented in the gene bank and with limited amounts of germplasm. Therefore, the gene bank manager must sample animals with the potential to yield the greatest number of offspring from the germplasm stored.

- Before the final decision about the animals that will be chosen as donors, all candidates should be submitted to a clinical and andrological or gynecological evaluation. During this evaluation a special attention should be given to animals selected as donors for their genetic traits but that may present sub-fertility.
- When choosing female donors, the ones with a good reproductive history should have a higher priority than the ones that have a history of poor reproduction or have never given birth.
- When choosing male donors, priority should be given to the ones that are known to produce good quality semen after freezing/thawing procedures.
- Both male and female donors must present morphological and behavioral characteristics to facilitate the collection of genetic material.

6.4.3. Sanitary aspects

Also of critical importance is that the conserved germplasm does not transmit pathogens into the future along with its genetic information. Thus, strict sanitary standards should be followed.

- Donor animals should be clinically inspected to confirm that it is healthy and free of contagious and infectious diseases. They should fulfil all requirements established by OIE in terms of infectious and contagious diseases that may be transmitted through semen and/or embryos (see Chapter 9). To meet all of the OIE standards, quarantine of animals to confirm health status prior to collection of germplasm may be necessary.

6.5. Collection of complementary biological material

In addition to the issue of germplasm collection of breeds critical to national food security, the gene bank has an opportunity and responsibility to collect germplasm or tissue samples for DNA analysis or other research purposes. It is suggested that this type of activity can be executed at the same time that other germplasm collection activities are ongoing. For example, it takes little additional effort to collect blood samples for health tests and future DNA analysis at the time of germplasm collection. Such efforts will in the long term increase the utility of the overall collection maintained by the gene bank.

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7. BASIC PRINCIPLES OF CRYOPRESERVATION

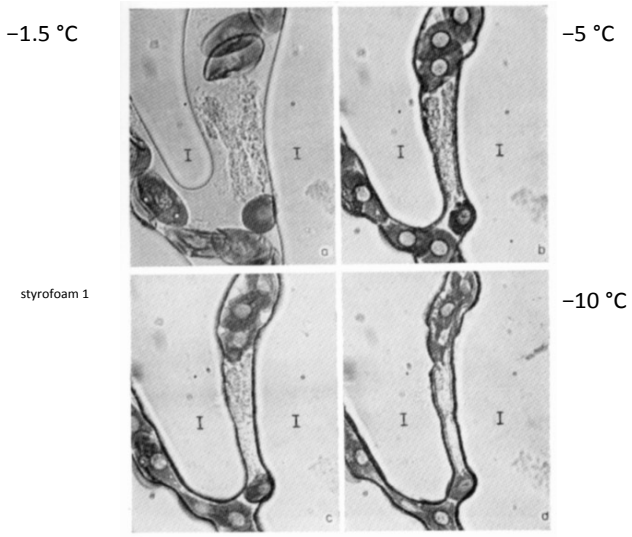
Spermatozoa were the first mammalian cells to be cryopreserved successfully (Polge *et al.,* 1949). This success was due to the serendipitous discovery by Polge and co-workers of the cryoprotective effect of glycerol. Since then, many methods have been developed for various types of cells, tissues and organs. Much progress in the field has come from empirical work as well as from fundamental cryobiology. Increased understanding of the causes of cryoinjury has continually helped to improve methods for cryopreservation. Furthermore, research into fundamental cryobiology has provided the basis for new cryopreservation concepts, such as vitrification.

7.1. Cryopreservation methods

Two important cryopreservation concepts are slow-freezing and vitrification. These are quite different concepts, but relate to the same physico-chemical relationships. The differences between the two concepts can be explained by first describing what happens during slow freezing.

In slow-freezing methods, cells in a medium are cooled below the freezing point. At some stage, ice formation will take place. The ice masses that form contain pure crystalline water. What remains between the growing masses of ice is the so-called *unfrozen fraction*, in which all cells and all solutes are confined (see Figure 7.1). The concentrations of the sugars, salts and cryoprotectant (e.g., glycerol) increase, while the volume of the unfrozen fraction decreases. The increase of the osmotic strength causes an efflux of water from the cells. Slow cooling is needed in order to allow sufficient efflux of water in order to minimize the chance of intracellular ice formation. As cooling continues, ultimately, the viscosity of the unfrozen fraction becomes too high for any further crystallization. The remaining unfrozen fraction turns into an amorphous solid that contains no ice crystals. This formation of a solid in the absence of crystallization is referred to as “glass” formation or vitrification.

Figure 7.1. Frog erythrocytes in the ‘unfrozen fraction, which is enclosed by growing masses of ice. (Rapatz and Luyet, 1960).



So, if cells in slow cooling methods become ultimately vitrified, what is different in *vitrification* methods? In vitrification methods, vitrification is achieved by using a medium with very high solute concentration to begin with, such that ice formation cannot occur in the entire sample. As ice formation does not occur, it is not necessary to cool slowly. In fact, in vitrification methods it may be beneficial to cool very rapidly. In both slow-freezing and vitrification protocols, the vitrified state and the physico-chemical conditions associated with it are to some extent similar, but the roads to get there are quite different.

7.2. Slow freezing

7.2.1. Chilling injury and cold shock

The first challenge in cryopreservation protocols for cells from homeotherm (i.e. warm-blooded) animals in is the cooling below body temperature. Cells may be damaged by very rapid cooling (cold shock) or may be damaged by the low unphysiological temperatures *per se* (chilling injury). Behaviour and function of membrane lipids and -proteins may depend on temperature. Membrane lipids that would normally be in a liquid crystalline state for instance may solidify at non-physiological temperatures, which can lead to changed function and begin processes such as cryocapacitation the production of Reactive Oxygen Species (ROS) that increase the damage to membranes. Decreasing the temperature may affect the rate of one process stronger than that of another process, which may cause an imbalance in cellular processes. One example is the disintegration of the metaphase spindle of oocytes, caused by a change in the dynamic equilibrium of association/dissociation of the tubulin filaments.

7.2.2. Supercooling

In slow freezing methods, cells will be brought in a suitable freezing medium and cooling is continued below the freezing point of that medium. Ice formation does not necessarily start at the freezing point. Small ice crystals have a lower melting/freezing point than “bulk” ice, due to their large surface tension. Spontaneous ice nucleation will in most cases occur after the solution is supercooled to a temperature between -5 and -15°C. Thereafter, ice will grow rapidly in all directions, and the release of the latent heat of fusion causes the sample to warm up abruptly, until the freezing/melting temperature of the solution (of the remaining unfrozen fraction) is reached. At this point, the ice formation stops, or will proceed at a rate then governed by the rate at which the heat of fusion is transported from the sample. Finally, the sample can “catch up” again with the lower temperature in the freezing apparatus. From a practical perspective, this all means that the cells undergoing cryopreservation in a typical semen straw have to withstand a series of large and abrupt temperature changes.

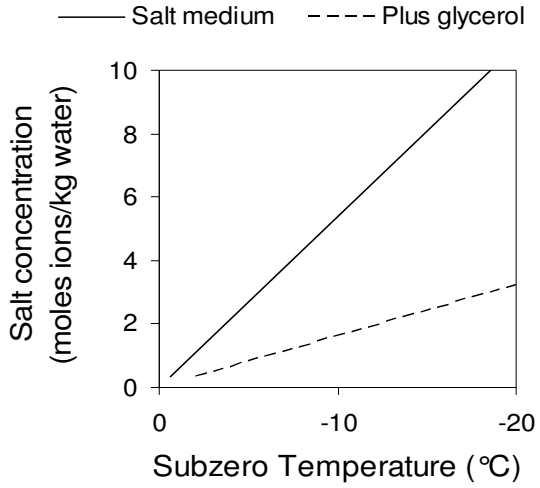
7.2.3. Conditions in the unfrozen fraction

Cells are faced with very high concentrations of solutes in the unfrozen fraction. The dehydration and high salt concentration could result in loss of stability of the membranes or denaturation of proteins (Tanford, 1980; Crowe and Crowe, 1984; Hvidt and Westh, 1992; Lovelock, 1953). Also, at these high salt concentrations, extracellular salts may enter the cells (solute loading, Daw *et al.,* 1973; Griffiths *et al.,* 1979). The fast efflux of water causes a rapid decrease of the volume of the cells to approximately 50% of their original volume, which leads to structural deformation of the cells. Further mechanical stress could come from being confined to very narrow channels of unfrozen solution, and squeezed in between growing masses of ice (Rapatz and Luyet, 1960).

7.2.4. The influence of cryoprotectants

At all practical cooling rates, the total solute concentration (in moles per kg water) is only determined by the subzero temperature (Figure 7.2). When the initial freezing medium only contains salts (electrolytes), extremely high salt concentrations will be reached in the unfrozen fraction. However, in a medium that contains a large proportion of non-electrolytes, at each sub-zero temperature, the total solute concentration will be the same as in a medium that contains only salts, but the salt concentration will be much lower.

Figure 7.2. The total solute concentration (salts plus non-electrolytes) is a function of subzero temperature. The presence of non-electrolytes like glycerol, therefore results in a lower salt concentration in the unfrozen fraction and inside the cells (Mazur and Rigopoulos 1983).



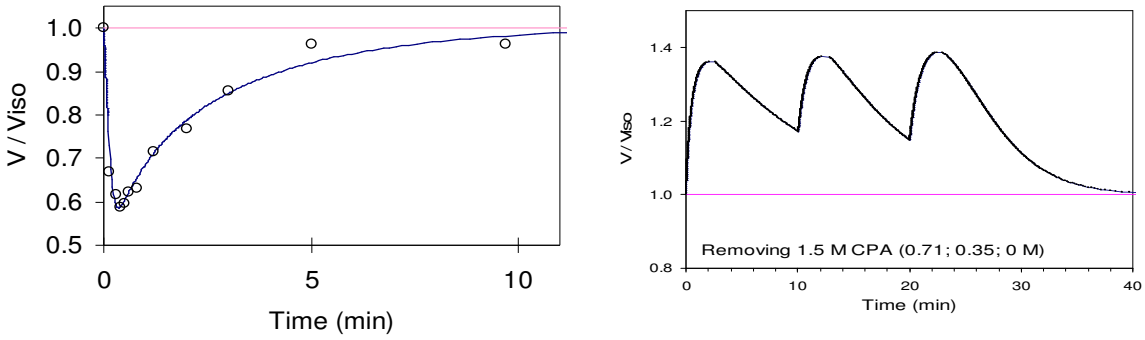
Sugars can be used as non-electrolyte solutes, but these will only affect the extracellular salt concentration. Moreover, high concentrations of impermeable solutes impose osmotic stress on the cells already before freezing. This is much less the case when a membrane permeable solute, such as glycerol, is used rather than a non-permeable solute. When cells are brought in a hypertonic glycerol medium, water will leave the cells because of the osmotic pressure difference. However, at the same time, glycerol will enter the cells. After a short time of equilibration, the cells will have regained their original volume. The osmotic stress imposed by a hypertonic glycerol solution is therefore much smaller, compared to a hypertonic sugar solution. Hence, glycerol may be used at greater concentrations than sugars, without damaging the cells. A substantial initial glycerol concentration in the medium means that part of the extracellular and intracellular water is replaced by the glycerol. Hence, the amount of ice formed is lower, the unfrozen fraction remains larger, the degree of shrinkage of the cells is limited, and the electrolyte concentration in the unfrozen solution and in the cells will be relatively small (see Figure 7.2). The mechanisms of cryoprotection by other membrane permeable substances, like ethylene glycol, DMSO, etc, are similar to those of glycerol.

There are additional mechanisms of cryoprotection by polyols like glycerol and several sugars. These substances can stabilize lipid membranes by hydrogen bonding with the polar head groups of membrane lipids (Crowe and Crowe 1984; Crowe *et al.,* 1985), which is especially important under severely dehydrated conditions. Secondly, these substances may affect the mechanical properties of the unfrozen fraction, especially its viscosity and glass-forming tendency.

The degree to which cells shrink and re-swell after addition of a membrane-permeable cryoprotectant depends on the concentration of the cryoprotectant and the ratio of the membrane permeabilities for water and for the cryoprotectant, respectively (Kleinhans, 1998). For instance, bull sperm shrink very little when brought in freezing medium with glycerol (Chaveiro *et al.,* 2006), whereas bovine embryos react much more strongly (see Figure 7.3, left panel).

After thawing, after removing the cryoprotectant, cells will do the opposite: they will first swell and then shrink again. This may lead to damage when the cells expand too much. This may be prevented by introducing step-wise removal of the cryoprotectant (Figure 7.3, right panel).

Figure 7.3. Volume excursion (shrink swell cycles). Left panel: Bovine embryos after addition of glycerol (5%v/v) (Woelders et al., 2007). Right panel: Stepwise removal of cryoprotective agent from1.5 M, 0.71 M, 0.35 M to 0 M. Simulated using a mathematical model (Woelders, unpublished).



7.2.5. The influence of cooling rate

A general observation in cryopreservation of cells and other biological systems is that each system has a specific optimal cooling rate, with decreased survival at cooling rates that are too low (slow cooling damage) and too high (fast cooling damage) (Mazur *et al.,* 1972).

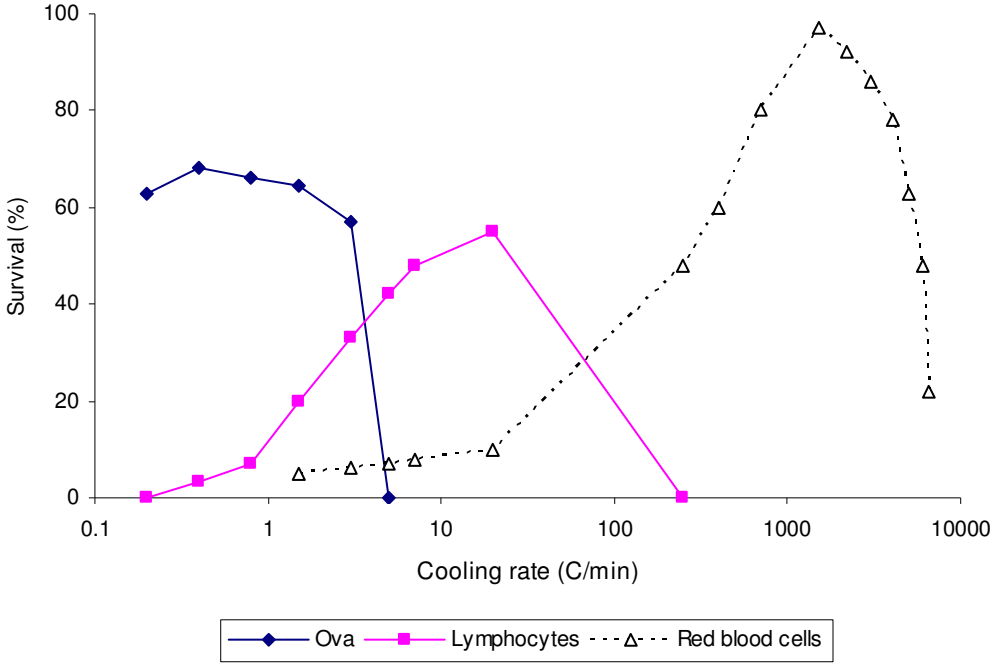
Ice growth is a rapid process, but transport of water through the cell membrane is a relatively slow process, because the membrane poses a resistance barrier. Therefore, as cooling and extracellular ice growth continue, the liquid water of the unfrozen fraction remains very close to equilibrium with the ice, but the intracellular water lags behind. This means that the water concentration (i.e. the chemical potential of water) is too high for thermodynamic equilibrium, and there may be a risk for intracellular ice formation.

When cells are cooled very slowly, the intracellular water lags behind only a little, and the risk of intracellular ice formation is minimal. However, it also means that the dehydration of the cells is maximal.

At higher cooling rates, intracellular dehydration, intracellular solute concentration and shrinkage of the cells will be less excessive, and, in addition, the cells are exposed to the unfavourable conditions for a shorter period of time.

When cooling rates are increased too much, however, the dehydration may not be fast enough to prevent the occurrence of intracellular ice nucleation (Mazur, 1963, 1977; Mazur *et al.,* 1972). There may also be additional causes of fast cooling damage. For instance, it has been proposed that rapid water flow through membrane pores could lead to an uneven distribution of pressure on the membrane (Muldrew and McGann, 1993, 1994). Also, fast cooling damage could result from the very sudden changes in size, shape and ultrastructure, caused by the rapid efflux of water (Woelders *et al.,* 1997). This means that there is a range of optimal cooling rates, which are neither too fast nor too slow. Different cells or other biological materials (embryos, tissue pieces) may have different optimal cooling rates. The optimal cooling rate of cells is largely determined by their volume and their membrane surface area (volume to surface area ratio), and by the membrane permeability of the membrane for water and for cryoprotectant. (see Figure 7.4.)

Figure 7.4. Cells may have a specific optimal cooling rate, showing a decreased survival at too low cooling rates (slow cooling damage) and at too high cooling rates (fast cooling damage). (Mazur 1985).



7.2.6. Interactions of cooling rate with thawing rate and cryoprotectant concentration

The optimal cooling rate may depend on various other factors, such as the cryoprotectant concentration and the thawing rate. It has been observed in semen from a number of species that the combination of fast cooling and slow thawing is particularly damaging to the cells. (Rodriguez *et al.,* 1975; Fiser, 1991; Henry *et al.,* 1993; Woelders and Malva 1998). If intracellular ice nucleation occurs at a low temperature and cooling proceeds rapidly, it could be that the cytoplasm turns into a glass before the intracellular ice crystals grow to a significant size, thus causing only sublethal, or no damage. During slow thawing, these small crystals can grow and subsequently damage the cells (Rall *et al.,* 1984). In addition, cells may be damaged by extracellular restructuring of ice masses called recrystallization (Bank, 1973).

7.2.7 Programmable and non programmable freezers

Biological material can either be frozen with quite simple non-programmable freezers or with more sophisticated programmable freezers (See Figure 7.5). Although programmable freezers are more expensive, they do not necessarily yield more satisfactory results, especially for experienced technicians and cryobiologists. Therefore, the choice between programmable and non-programmable systems will depend on the financial resources available and the experience of the technician. In some cases, even the most experienced technicians nonetheless prefer the operating simplicity of the programmable models.

Figure 7.5. Examples of programmable freezers. These freezers are examples from IMV technologies® of France.

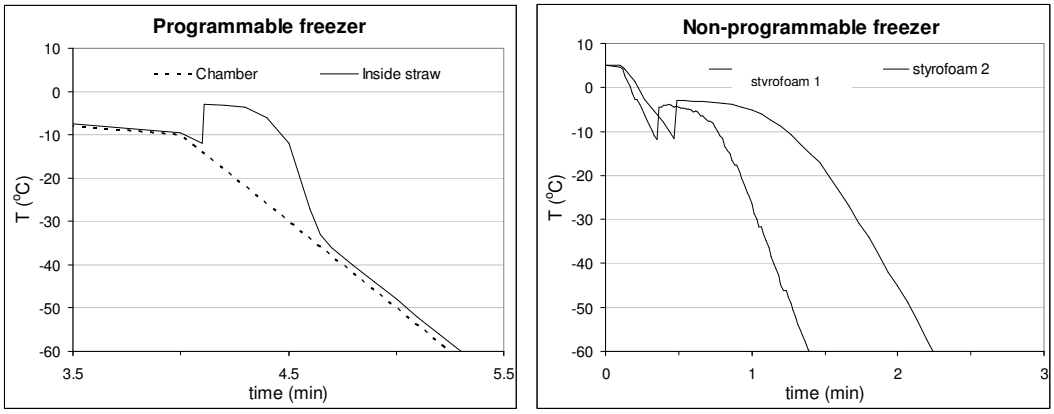
In most programmable freezers, the straws or vials are cooled by cold nitrogen vapour. The temperature inside the cooling chamber can be accurately controlled and the time course of that temperature can be programmed to follow a chosen time course. However, the time course of temperature inside the straws may be different due to the generation of heat of fusion. (Figure 7.6)

In non programmable freezers, the straws may be cooled by being exposed to vapour (or a cold surface) at a constant low temperature. An example of a simple system is the freezing of straws placed on a rack in a styrofoam box partially filled with LN2 without ventilation. The height of the straws above the LN2 then determines the rate of heat exchange. Alternatively, straws may be placed on a piece of styrofoam that floats on the LN2 (e.g. Dong *et al.,* 2009). The thickness of the styrofoam piece then determines the rate of heat exchange.

Generally, in such systems, the rate of heat exchange is governed by the difference between the temperature inside the straw and that outside the straw, and the heat conduction, while the latter strongly depends on volume surface ratio of the straw or vial and the rate of (forced) ventilation. Therefore it is difficult to compare one type of non-programmable freezer with another, or to actually know the freezing rate obtained with any given non-programmable apparatus. Experimentation is needed to determine which conditions are optimal.

However, such non-programmable systems have an advantage. The cooling curve (the time course of cooling and freezing) is by default of the form theoretically predicted to be optimal for slow freezing (Woelders and Chaveiro 2004), with relatively low cooling rates directly after initiation of ice formation and higher cooling rates later on. The bulk of the ice formation happens in the temperature range between the freezing point and -10°C, and consequently most of the water efflux from the cells must take place in that temperature range as well. Thus, the heat of fusion liberated during ice formation slows down the cooling exactly when cells need the extra time to export intracellular water. The overall steepness of the freezing curve can be adjusted in such non-programmable systems by choosing the height of the straws above the LN2 (= the temperature of vapour around the straws) (Figure 7.6). In more sophisticated systems with forced ventilation and with adjustable preset vapour temperatures, the rate of heat exchange can be adjusted by choosing the preset vapour temperature.

Figure 7.6. Left panel: A typical freezing programme in a programmable freezer (dotted line) and the corresponding freezing curve measured inside the straw. Note that the temperature inside the straw doesn't necessarily follow the programmed chamber temperature and that the cooling rate in parts of the curve may be higher than anticipated. Right panel: In a non-programmable freezer (styrofoam box), the constant temperature outside the straw results in sigmoidal freezing curves. The overall steepness of the freezing curve can be changed by choosing a different vapour temperature, e.g. by changing the height of the straws above the liquid nitrogen (Woelders and Zuidberg, unpublished).



7.3. Vitrification

7.3.1. Chilling injury and cold shock

As for the slow freezing methods, cold shock and chilling injury could injure cells or tissues to be vitrified. Depending on the material and the protocol, cells or tissues may be rapidly cooled from a temperature at which chilling injury and cold shock play no role, e.g. room temperature. Extremely high cooling rates from that temperature to the glass state seem to be able to "outrun" cold shock and chilling injury. One example is that rapid cooling seems to be able to prevent disintegration of the metaphase spindle of oocytes.

7.3.2. Cryoprotectants

In vitrification methods, cells or tissues are brought into a medium with a very high concentration of CPA. If the concentration of solutes is high enough, vitrification solutions will solidify to a glass without any risk of intracellular or extracellular ice formation during cooling or warming, independent of the cooling and warming rates used. However, the very high concentrations of CPA needed for vitrification may cause damage due to abrupt osmotic changes, the extremely low water potential, or chemical toxicity. In the description by Rall (1987), the embryos are first equilibrated with 25% vitrification solution (VS) at room temperature. Then, the embryos are cooled to 4°C and transferred to 50% VS and 100% VS and then rapidly packed and transferred to LN2. The stepwise increase of CPA concentration reduces osmotic effects, while the lower temperature and the shorter duration help prevent damage by chemical toxicity. In addition, the chemical toxicity may be decreased by using mixtures of various permeant CPAs, or addition of non-permeant CPAs (60 g/l polyethylene glycol (Rall, 1987), or 60 g/l BSA (van Wagtendonk-de Leeuw *et al.,* 1997).

7.3.3. Reduction of CPA concentration at high cooling rates

Solutions that have a solute concentration lower than that of classical vitrification solutions have a freezing point below which there is a significant tendency to form ice crystals. But when the solution is cooled very rapidly, there is simply no time for ice formation. Below a certain temperature, the solution becomes so viscous and stiff that ice formation becomes impossible, and

the solution turns into a "metastable" glass. The solute concentration needed for metastable vitrification decreases as a function of increasing cooling rate. The most recent vitrification procedures therefore make use of high cooling rates, in order to reduce the concentration of CPAs to decrease damage due to osmotic stress and chemical toxicity.

The cooling rate may be increased in several ways. One is to reduce the volume of the sample to be vitrified. An early example of this approach is the Open Pulled Straw (OPS) method (Vajta *et al.,* 1998, 2000). Even smaller sample volumes were used on electron microscope grids, so-called hemi-straws, nylon loops (cryoloops) or polypropylene strips (cryotop) (Kuwayama, 2007; (Kitazato Supply Co., Fujinomiya, Japan). The cryotop system allows one to vitrify a volume of 0.1 µl.

In addition to reducing the sample volume, an increase of cooling rate can be achieved by heat transfer to a liquid that does not boil. Liquid nitrogen at its boiling point (−196°C) will generate nitrogen gas when it absorbs heat. This will lead to a film of gas that insulates the sample from the LN2. LN2 at its freezing point (also known as "nitrogen slush") doesn't have this disadvantage. It can be produced with an apparatus called Vit Master® (IMT Ltd, Ness Ziona, Israel) (Arav, 2002).

In these metastable vitrification procedures, it is essential that also the warming (i.e. thawing) of the sample proceeds at a very high warming rate. If warming were to be done slowly, ice crystals could form in the temperature range between the vitrification temperature and the freezing point of the vitrification solution.

Most recent vitrification protocols make use of these ultra rapid approaches to reduce CPA concentrations and to prevent cold shock and chilling injury. Current vitrification solutions (Liu *et al.,* 2008; Morató *et al.,* 2008) have much lower solute concentrations than those used in classical vitrification solutions (e.g.such as VS3, Rall 1987). As described in Chapter 4, very good results are currently obtained when using these approaches for vitrification of oocytes and embryos. Recent studies with swine and cattle oocytes have indicated that the Cryotop systems gives superior results compared to the OPS system (Liu *et al.,* 2008; Morató *et al.,* 2008).

7.4. Freeze drying

Storage of freeze-dried biological material is extremely cost efficient, as no expensive and bulky LN2 containers are necessary. Furthermore, it is safe. The material may be stored at ambient temperature and there is no risk of malfunction of equipment or personal injury from LN2, as is the case in cryogenic storage. On the negative side, however, freeze drying generally results in loss of cell viability. Therefore, standard insemination procedures cannot generally be used for freeze-dried sperm. However, freeze-dried sperm have been successfully used for ICSI to give live offspring in mice and rabbits (Wakayama and Yanagimachi 1998; Liu *et al.,* 2004). In addition, freeze-dried somatic cells have been successfully used for SCNT to produce apparently healthy embryos (Loi *et al.,* 2008a, 2008b). There have been no cloned offspring by SCNT using freeze-dried somatic cells reported so far, however. Therefore, this technology has the potential be useful for gene banking of genetic resources to regenerate live animals and recover lost breeds, although this would require further development and optimization. But surely, freeze-dried gametes and somatic cells can already be used for conservation of genotype collections for (genetic) research purposes.

The key to freeze-drying is that the material is brought to a vitrified glass state in which the glass transition temperature is increased to a point above ambient temperatures. The first phase requires that the biological material is brought to a vitrified state. Vacuum is then applied, resulting in sublimation of any ice that may be present) and in a further decrease of the water content of the vitrified material to increase the glass transition temperature, ultimately to a level greater than ambient temperatures. Therefore, the material can be stored at ambient temperature while remaining in the stable glass state. Obviously, the initial freezing/vitrification procedure and the medium used should be optimal to ensure the survival of the germplasm throughout that initial

phase. Secondly, the medium composition must be optimal to prevent damage of the cells from effects of the subsequent further dehydration of the material.

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8. COLLECTION OF GERMPLASM AND TISSUES

Germplasm collection can occur on farm or at collection facilities depending upon the within country conditions, resources and availability of the animal populations. Collection and processing procedures will differ widely depending upon the type of germplasm being collected and the donor species. This chapter presents an overview of collection procedures for the various types of germplasm, including information particular to each of the major livestock species. Further detail regarding germplasm processing and cryopreservation are presented in the Appendices.

8.1. Semen

Collection of bull, ram, buck, rabbit and stallion semen is typically performed using an artificial vagina (AV), whereas boar semen is usually collected by using the hand-glove method. These methods are usually performed on animals that have been trained for semen collection and are thus habituated to contact and interaction with humans and the collection equipment. Electro-ejaculation may be considered for animals that have not been properly trained, although it should be avoided for boars and stallions. If a male has died before sufficient semen has been collected or if a male can be sacrificed because its genetic material is considered more valuable than the live animal, epididymal semen can be collected directly from the testes. For poultry, the abdominal stroking technique can be used for semen collection. For a scientific review of semen collection methods for various mammals, see Watson (1978). For the basic supplies and equipment needed for semen collection and freezing, see Appendix B.

8.1.1. Collection with an artificial vagina (bull, ram, buck, stallion and rabbit)

Different types and sizes of AV are available according to the species and breed within species (particularly for stallions). Before collection, the AV should be prepared with a large-enough volume of warm water to ensure sufficient physical pressure to stimulate the glans penis of the male. The inner wall of the AV should be between between 42° and 48°C, depending on animal body temperature, and should remain within this temperature range throughout the semen collection process. The collection liner of the AV should then be lubricated with a nonspermicidal sterile gynaecological lubricant.

The use of a "teaser" animal of the same species is recommended for the collection process. The teaser animal facilitates collection by allowing the donor male to mount and object in a way similar to natural mating. A live teaser may also be used to increase arousal, by allowing the donor to follow the teaser while it is led around the collection area immediately prior to collection. For stallions, collection via AV is often performed by using a "phantom mare" rather than a live teaser animal. A phantom mare is a man-made object constructed to mimic the size and shape of a real horse. For rabbits, the teaser is typically a female and the AV is affixed at the vulva.

Once the teaser is readied, the donor male is allowed to mount one to two times for false collections. The false mounting procedure will allow the male to become aroused and increase the final volume of the ejaculate. During these false collections the penis of the male is diverted to protect the teaser animal and does not enter the AV.

Once aroused, the male is allowed to mount and the penis is guided to enter the AV. The AV should be held in a manner so that the height and angle approximate that of the donor male. The male is allowed to thrust and remain on the teaser until ejaculation is complete. The AV then is taken into the laboratory and the semen is processed for insemination or cryopreservation.

Safety of the personnel, donor and teaser animals should be a primary concern for semen collection with and AV, especially for large species. The teaser animal should be securely restrained. The collection area must provide secure footing for both the semen donor and teaser. For stallions in particular, protective head gear such as a helmet is needed for all persons handling the animals prior to, during and following collection.

8.1.2. Electro-ejaculation (bull, ram and buck)

In general, the AV method is preferred for semen collection, as it tends to yield the highest quality semen with the least stress on animals. However, in some situations where the male cannot be trained for conventional collection, such as at remote sites in the field, collection via electro-ejaculation is the most practical option.

Prior to the electro-ejaculation procedure the collection tubes (or cones) must be prepared. In the case of the bull, the conical glass tubes need to be insulated using a 37ºC water jacket. Ram and buck semen collection tubes can be handled similarly or simply kept insulated by the hand of the collecting technician.

The probes of electro-ejaculators differ in size according to species, as do the specific collection methods. In addition, operating instructions differ according to the manufacturer, so these instructions must be carefully followed. In addition, training and experience are needed to be proficient in semen collection using this methodology.

For cattle, electro-ejaculation is performed with the bull restrained in a standing position. The probe is lubricated and inserted into the rectum with the metal electrodes facing ventrally (downward). The electro-ejaculator is turned on and the voltage increased (manually or automatically) in small increments until the bull maintains an erection. The oscillating voltage peaks are then continued until semen is ejaculated and collected into a clean vessel.

For bucks and rams, the male is placed on his side and the penis is extended from the sheath by stretching the sigmoid flexure. The penis is grasped with sterile gauze and the glans penis (with its urethral process) is diverted into a 50-ml disposable tube. The lubricated electro-ejaculator is then inserted into the rectum of the animal and used to gently massage the accessory glands by exerting a downward pressure on the bottom of the rectum. This pressure should be applied for 10 to15 seconds prior to turning on the electro-ejaculator. After massaging, the electro-ejaculator is turned on for 3 to 8 seconds and then the animal is allowed to rest for 15 to 20 seconds. Massaging the accessory glands in between stimulation will cause the male to ejaculate. Stimulation is usually not performed more than three times during the collection process and at least 1 hour is allowed between collections. After ejaculation, the semen is covered to maintain its temperature and taken to the laboratory for processing.

8.1.3. Gloved-hand collection technique (boar)

For collection of swine semen, the boar is first allowed to mount a teaser animal or mounting dummy. The penis needs to be fully extended prior to semen collection. The protruding penis is then grasped so that the ridges of the penis are between the collector's fingers and pressure can be applied to the gland penis with the smallest finger of the collector's hand. After the initial fractions of the semen are ejaculated, the sperm rich portion (milky appearing portion) should be collected into a 37°C insulated container covered with two layers of sterile gauze to remove the

gel fraction. The remaining fraction is then ready for further processing. For an overview on methods of evaluating boar semen quality see Woelders (1991) or Colembrander *et al.* (2000).

8.1.4. Abdominal stroking (poultry)

In poultry, semen collection is performed by the abdominal massage method previously described by Burrows and Quinn (1935). This procedure works best when done by two persons working together. One person carefully restrains the male donor between his or her arms and body, while the second person collects the semen. This person massages with firm rapid strokes the abdomen of the bird from behind the wings towards the tail. The behaviour of the animal indicates its readiness to ejaculate as the male responds with the tumescence (erection) of the phallus. At this point, the handler gently squeezes the cloaca with two or three fingers, expressing semen through the external papillae of the *ducti deferentis* and into a pre-warmed tube held underneath the cloaca. The person collecting the semen must be careful, because the close proximity of the penis and cloaca increases the likelihood of semen contamination with feces, urates and bacteria that have a detrimental effect on semen quality.

Semen may be collected from mature males twice or three times per week. Either a glass graduated funnel-shaped tube or just a standard graduated glass tube may be used. Preferably the semen extender (e.g. Lake extender – Lake, 1960) and the collection tubes are placed in a 25°C incubator for processing.

8.1.5. Epididymal sperm collection (mammalian species)

Attempts have been made at harvesting epididymal sperm from the live intact farm animal males over the years by either catheterization or flushing the lumen of the cauda (tail) epididymis with a hypodermic needle and a plastic syringe. Of these two approaches, the best success has been reported with catheterization of the *cauda epididymis* in the mature male. In most males, the catheterization procedure is successful but often post-surgical problems with the indwelling catheter have caused this approach remain relatively unpopular.

The most often used approach to harvest epididymal sperm is to surgically remove the testes from the male before or shortly after death. With this approach, the excised testes are placed in a sterile resealable (zip-lock) plastic bag, then into an empty styrofoam cooler to maintain them at body temperature and transported to the laboratory for processing. For longer distance transport, the testes can be chilled and the temperature during transport is usually adjusted by placing ice or ice packs in the bottom of the cooler. The temperature of farm animal testes upon arrival at the laboratory should not be below 5°C before processing, however.

The most common method of harvesting sperm is by slicing (with a scalpel) across the cauda portion of excised epididymis that has been placed in a sterile petri dish containing a sperm medium (Guerrero *et al.,* 2008, 2009). A second approach is to make two incisions in the cauda and use 30-ml syringe attached to a plastic tube to flush the sperm from the lumen into a dish with a retrograde flow of sperm medium (see Saenz *et al.,* 2008). The *caudae epididymides* of goats can be sliced and suspended in Salomon's one-step freezing medium at a 1 to 4 dilution rate. Then, after 2 filtrations (75 and 50 µm) through a nylon grid at room temperature, sperm suspension is then free from any somatic cells and is ready for further processing (Ehling et al., 2006). The intact testes of some animals (e.g., dog) can be frozen (-20°C) in a plastic Zip-Lock bag shortly after collection and allow viable sperm to be collected after thawing the testes (Graff *et al.,* 2000).

8.1.6 Shipping and processing of collected semen

Following collection, the samples can be immediately cryopreserved or transported to a laboratory (up to 24 hours holding time) for cryopreservation. The protocols for processing are species-specific and thus, require specific media as well. Details about processing and freezing samples are presented in Appendix C, according to species. Evaluation of semen is an essential aspect of semen processing, to prevent the freezing of semen that is not viable and to quickly

identify possible problems with semen donors. Appendix D provides general guidelines for semen evaluation, which are valid for all livestock species.

When collection occurs on-farm or in the field, semen samples must be maintained in a temperature controlled environment during transport to the processing centre. A shipping container should therefore be prepared immediately prior to collection of the semen. The standard shipping container has both an inner and an outer styrofoam box. The outer box will hold numerous sealed reusable frozen ice packs and a small styrofoam box.

The ice packs are placed in the outer box prior to sample collection and following collection of the semen samples they are placed in the inner box. For bulls, rams and goats the quantity of ice packs must be sufficient to cool the samples to 5°C; for stallions and boars, the samples need to be cooled to 15°C. The number of ice packs necessary will have to be determined on a case-by-case basis, as all types of commercial ice packs provide different amounts of cooling ability due to size and volume.

8.2. Embryos

Production, collection, processing and freezing of embryos is a more demanding process than for semen and a greater level of training and experience is required. The following subsections will address the major issues of importance in cryoconservation of embryos. FAO has previously produced manuals for ET for several species, including FAO Animal Production and Health Papers No. 77 for cattle[13], No. 84 for buffalo[14] and No. 115 for sheep and goats[15]. In addition, commercial manuals are also available for purchase, such as by the International Embryo Transfer Society (IETS - www.iets.org).

8.2.1. Superovulation of donor females

Ideally, to maximize N_e in a reconstituted breed, each female embryo donor will produce at least one male and female offspring. To reach this goal, each female will obviously need to produce multiple embryos. To increase the number of embryos per collection, donor females are administered various hormone agents (gonadotropin-like) to stimulate the ovaries to produce to multiple ova for fertilization and embryos for collection. Early reports in cattle, sheep, goats and swine used Pregnant Mare Serum Gonadotropin (PMSG) at various dose levels (units) to superovulate donor females. PMSG is extracted from the serum of pregnant mares after 40 days of gestation and has potent follicle stimulatory activity from a single intramuscular injection. For information on the use of this agent in donor cattle see reviews by Elsden *et al.* (1978) and Saumande *et al.* (1978). This agent (now termed equine chorionic goandotropin or eCG) is still the agent of choice in swine and often the choice in sheep and goats. eCG has a long half life, however, and often over stimulates the ovaries of donor cattle. Therefore, eCG is no longer the agent of choice in North America, although it is still being used in countries where other gonadotropic agents are not commercially available.

Today, Follicle Stimulating Hormone (FSH) has become the agent of choice for superovulating donor cattle and is now also being used in sheep and goats. FSH has a much shorter half life in the circulation and thus, is usually administered by twice daily injections for 3 up to 5 days (see Monniaux *et al.,* 1983; Armstrong, 1993; Mapletoft *et al.,* 2002). However, success has also been reported in cattle by administering once daily injections (e.g. Looney *et al.,* 1981; Bo *et al.,* 1994). *Bos indicus* cattle appear to be more sensitive to FSH than do *Bos taurus* cattle. Various modifications on approaches to superovulating *Bos indicus* cattle have been developed and are now in use (see Baruselli *et al.,* 2006, 2008; Bo *et al.,* 2008).

Information on various superovulation procedures are presented in the FAO documents No.77 for cattle[1] and No.84 for buffalo[2]. Some of the more commonly used superovulation schemes for

[13] http://www.fao.org/DOCREP/004/T0117E/T0117E00.htm

[14] http://www.fao.org/DOCREP/004/T0120E/T0120E00.htm

[15] http://books.google.com/books?isbn=9252033882 (French).

cattle donors today are presented in Appendix E. For successful embryo recovery, the current recommendations for number of inseminations and the number of units of semen needed per donor cow for optimum fertilization are 1 or 2 inseminations with 1 or 2 units of good quality semen per insemination (see Schiewe *et al.,* 1987).

It is very important to select the appropriate number of embryo donor females to match each sire in the breeding schedule to improve rates of genetic variability in each cryopreservation programme (e.g. Woolliams, 1989). Chapter 4 presented data (Table 4.1) that gave an indication of the number of transferable embryos that may be obtained after a single superovulation treatment and embryo recovery, as well as estimates of the number of embryos achievable for one donor female during one year. However, the responses across animals are quite variable. Some females simply do not respond or stop responding to the stimulatory agents, or develop physiological conditions that make it difficult to retrieve the embryos. Thus, although 25 donor females and 25 donor males is the recommended minimum, a larger number of candidate females may be needed, because of the likely failure to obtain embryos from some donors. The expected rates of success in both collection and transfer must be considered when determining the number of embryos to collect and store. With experienced ET professionals, the cattle embryo recovery rates are expected to be >75%, with 4 to 8 good quality bovine embryos per donor collection. Using good quality embryos for transfer, 65 to 80% pregnancy rates are now expected in well-managed cattle operations. Expected pregnancy rates from embryo transfer for a variety of farm animal species were presented in Table 4.2.

8.2.2. Stages of embryo development

Embryos continually develop through various morphological stages after *in vivo* fertilization. As the embryos divide the number of embryonic cells (blastomeres) increase per embryo as they migrate through the reproductive tract of the female (Table 8.1). It is important to know when the embryos are supposed to be in the uterus of the superovulated female, so that nonsurgically embryo recovery can be performed to obtain the embryos from the uterine horns.

Table 8.1. Location of the embryo at different time points following estrus.

Day[a]	Morphological Stage	Location
1	Fertilized ovum	Oviduct
2-5	2- to 16-cell stage	Oviduct
3-4	Early morula	Oviduct
4-5	Compact morula	Distal uterine horn
5-6	Early blastocyst	Distal uterine horn
6-7	Blastocyst	Distal uterine horn
7-8	Expanded blastocyst	Distal uterine horn
8-9	Expanding hatched blastocyst	Distal uterine horn
9-10	Hatched blastocyst	Uterine horn[b]

[a]Days from standing estrus.

[b]After hatching the embryo begins migration towards the middle portion of the uterine horn.

An embryo technician must be able to recognize not only the stage of embryo morphological development but also to assess embryo quality before selecting and transplanting embryos. This ability to make this judgment can be developed only with experience gained in assessing and grading embryos in the laboratory. For a review on assessing embryo quality and classifying embryos see the classic training publications by Lindner and Wright (1983) and Robertson and Nelson (1998).

8.2.3. Embyro collection

Farm animal embryos are collected from a donor female by flushing the reproductive tract using a physiological flushing medium. The most often used flushing medium for cattle is phosphate-buffered saline (PBS), which can be mixed from commercially available dry packets and water or

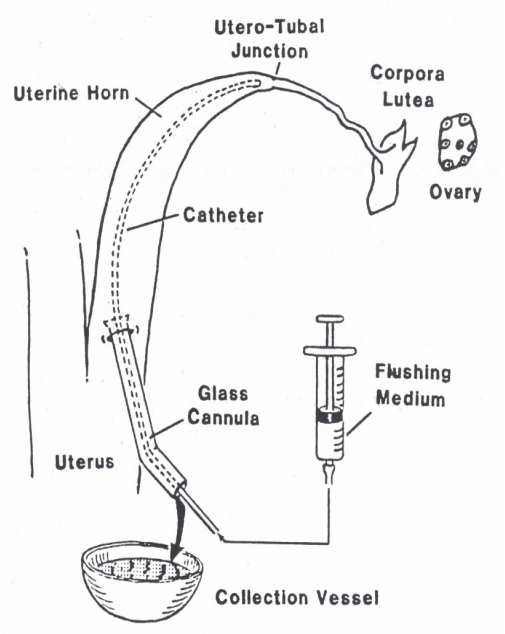
similarly purchased as a ready prepared solution. Various other media are also commercially available. Harvesting donor embryos is most often done by a nonsurgical standing method in some species such as cattle, horses and buffalo but usually requires a surgical approach in other species such as pigs, sheep and goats (see review by Betteridge, 1977).

8.2.3.1. Surgical embryo collection

Today, surgical embryo collections for swine, sheep and goats are usually done at either at a commercial ET transplant units. Information on surgical procedures available is presented in the FAO document No. 115 for sheep and goats[16]. In addition, see Kraemer (1989) or Baldassarre and Karatzas (2004). Over the years various research reports have described various nonsurgical approaches for embryo collection and transfer in these species (see reviews by Foote and Onuma, 1970; Betteridge, 1977), however, in most cases, the number of embryos recovered per collection and the pregnancy rates per embryo transferred are reduced when compared with the standard surgical approaches.

Figure 8.1 is a simple diagram of the surgical collection procedure for sheep and goats. The procedure is typically performed laparoscopically, which the insertion of a glass cannula and catheter into the uterus. The flushing of the uterus by injection of an appropriate media with a syringe attached to the catheter. The injected liquid causes the embryos to flow out of the uterus into the collection vessel, at which point the embryos can be identified, processed and cryopreserved.

Figure 8.1. Commonly used approach to collect embryos (morulae and blastocysts) from sheep and goat donor females (drawing courtesy of R A. Godke).



Embryo collection in pigs is also usually done surgically. Given that pigs naturally produce multiple offspring, superovulation is usually not practiced, as embryo viability may be reduced. Collection is performed at 5 to 8 days after insemination, via laparotomy at a midventral position. For this procedure, the female is maintained under general anesthesia. At this stage, embryos will be in the blastocyst stage. Collection can be done earlier, in the 4 to 8 cell stage, but will then require further culture before transfer and additional manipulation, such as delipidation.

[16] http://books.google.com/books?isbn=9252033882 (French).

8.2.3.2. Nonsurgical embryo collection in cattle

Today, virtually all cattle embryos collected in-field and in-clinic by commercial ET companies are collected by a simple, noninvasive nonsurgical procedure (see FAO, 1991a). Nonsurgical embryo collection and transfer poses little risk to the cow, and greatly reduces the time needed for harvesting embryos. The drawback to nonsurgical embryo collection is that embryo recovery rates may be a little lower for the less experienced technician.

There are two basic approaches to nonsurgically recover embryos in cattle (see review by Chapman and Godke, 2004). For a listing of the equipment used in nonsurgical embryo collection and transfer procedures in cattle see Appendix F. The body of the uterus and uterine horns can be flushed simultaneously using one flushing procedure, often called "Uterine Body Flushing" or "Body Flushing" or each uterine horn can be flushed separately using two flushing procedures, which is called "Uterine Horn Flushing" or "Horn Flushing". The flushing of donor cattle with either of these approaches usually recovers 50 to 90% of available ova/embryos, depending on the experience of the technician conducting the procedure. The potential number of embryos available for collection can be determined through palpation or ultrasonic examination based on the number of *corpora lutea* (CL) present on the ovaries of the donor female. Rectal palpation of donors with a large number of ovulations yields a rather imprecise estimate, however. It is therefore recommended that ultrasonography be used to evaluate the ovaries of the donor prior to the embryo collection procedure, if possible.

When using the body flush procedure, a Foley catheter is inserted through the cervix and into the uterine body. The cuff is then inflated and pulled back against the internal os of the cervix. The catheter is connected to a "Y" connector that allows medium to flow in through the catheter out of the female into an embryo filter apparatus to capture the embryos. When flushing, the uterus and the horns are allowed fill with medium until turgid, then manually massaged to recover the embryos when the uterus is drained. This filling and draining process is repeated until the volume of flushing medium fluid allotted to the female is depleted. The body flushing procedure uses ~1 000 ml of flushing medium for each donor animal

With independent horn flushing, the catheter is passed through the cervix and into the uterine horn. The tip of the catheter should be placed anterior to the external bifurcation of the uterus ½ to ¾ of the distance through the lumen towards the distal tip of the uterine horn. The cuff of the catheter is then inflated and 750 ml of flushing medium is allowed to flow into the horn. Manual manipulation is then used to recover the embryos and the medium from the horn. When one horn has been flushed, the cuff is deflated and the catheter is removed and then placed into the contralateral horn and the same flushing procedure is repeated. For this approach, ~1 500 ml of medium is used per donor animal.

Hay *et al.,* (1990) conducted a comparative study between body and horn flushing for recovery of embryos. On average, a greater number of embryos were obtained through horn flushing, but the difference was not significant. Given that the difference between the two approaches to collecting embryos was not significant, the conclusion was that the method used for collection should be decided by the technician based on his or her preference and proficiency. A modification in the uterine flushing approach in cattle, termed the "shallow uterine horn flushing technique", has recently been reported to be successful in dairy heifers (Sartori *et al.,* 2003).

One should not overlook the potential use of a single embryo collection procedure (i.e. without superovulation) in cattle for on farm use. With this approach, the donor female can remain on the farm, thus reducing the risk of disease transmission and no ovarian stimulating agents are needed. This approach is expected to reduce stress on the donor females, allowing them to maintain a constant level of productivity. The collection method is the same as that for superovulated donor females, but there is usually less uterine endometrial swelling (from the hormone stimulating agents), resulting in an increased chance of harvesting the 7 or 8 day-old embryo by less experienced technicians. This approach may be particularly useful when reconstituting populations with a combination of stored semen and embryos or as part of *in vivo* conservation programmes, where many offspring per living female are not necessarily desired, inasmuch as

they would contribute to increased relationships among the animals in the live population. Sexed semen can be used to increase the probability of obtaining offspring of the desired sex.

Many factors may adversely affect recovery rates, such as poor nutritional status of the donor, improper (either over- or under-) hormonal stimulation of the donor, failure of the fimbria of the oviduct to pick up the ova, poor quality of semen used to inseminate the donor cow, failure of embryos to enter the uterus after fertilization and failure to collect the embryos during the flushing procedure. Many of these factors may be associated with inexperience of the technicians.

8.2.3.3. Nonsurgical embryo collection in horses

Successful ET with live offspring was first was reported in the horse in the early 1970s in England and Japan. In fact, the nonsurgical embryo collection and transfer procedures used today in the mare are easier to perform than in the cow. The basic nonsurgical collection and transfer procedures used today for the mare was reported by Colorado State University (Imel *et al.,* 1981), with several modifications subsequently reported to improve the ET procedure in the mare (see Wilcher and Allen, 2004). Furthermore, the latter-stage horse embryo is even large enough to see without the use of a microscope. Embryo collection and transfer technologies have been held back by rules and regulations of various breed associations in some countries, whereas in other countries the use of these technologies is increasing at a rapid rate. The use of ET in horses has become particularly common in Brazil.

Although the mare can be given hormones to superovulate her ovaries, donor mares tend to produce fewer oocytes for fertilization (range of 2 to 4) post-treatment than do donor cows. Usually, no more than one embryo is produced from a donor mare per cycle for potential embryo collection. To obtain early stage embryos (single ovulated, <300 microns in diameter) for cryobanking would require >130 mare cycles to harvest 100 embryos. By superstimulating the mares the number of mare cycles needed to produce the same number of embryos would likely be reduced somewhat.

8.2.4. General recommendations on embryo collection

To maximize efficiency, the collection, processing and storage of embryos must be carried out by trained professionals. In fact, many countries will have specific regulations on who can perform embryo collection. Technicians will need to undergo special training relating to sanitation and techniques.

Donor animals, if possible, should be subject to quarantine and/or health testing prior to collection. At the time of collection, the donor animals must also be kept as clean as possible and body parts that will be accessed and manipulated during the procedure (e.g. tail, vulval area) should be washed and dried. When surgical collection is performed, hair should be clipped from incision sites and the area must be washed, rinsed and disinfected. Animals must be well restrained and treated in a manner to avoid stress or other factors that may compromise the welfare of the donor.

The embryo collection team (usually two or three technical people) needs to have at its disposal either well-maintained, clean and sanitary permanent facilities or a mobile laboratory where embryos can be collected, evaluated, processed and packed. The processing laboratory needs to be clean and equipped with an appropriate working space, electricity, temperature-controlled incubator(s), microscope(s) and other technical equipment and supplies (see Appendix F). Small equipment must be sterilized between collections and single-use disposable materials are recommended for sanitation purposes, when possible.

The direct disease risk related to embryos, although being low, depends very much on the handling of the embryos by the embryo collection team. This entrusts a very high responsibility on a competent embryo collection team, to perform the collection, processing and storage of embryos according to the defined protocols. Given this important responsibility, and to ensure that the work is always done to the required high standard, it is recommended that a procedure for approving and officially recognizing members of these teams is introduced.

The potential health risk can be large when the recommended procedures regarding collection and handling are not precisely followed. It is very important review the IETS recommendations for the sanitary handling of *in vivo*-produced embryos before beginning embryo collection (see Stringfellow, 1998) as well as the OIE standards (See Chapter 9 and www.oie.it), especially in the latter case if gene banking involves transboundary movement of embryos.

Abundant results from world-wide research on the risks of disease transmission via embryos are available for the bovine. Less information is available for sheep, goats and swine and is almost nonexistent for the other species. Any embryo collection should be preceded by an extensive clinical examination for the presence of diseases of the donor animal, its herd or flock mates and the general environment in which the animals are kept. This clinical examination may also influence subsequent treatment for superovulation and recovery, as one can only expect good results from perfectly healthy animals.

The disease risks may vary among species but this should not influence the level of attention. More information can be found in FAO Animal Production and Health Paper No. 23 on Disease Control in Semen and Embryos[17]

8.2.5. Conventional embryo freezing

Embryos are usually frozen when they are at the morula or blastocyst stage, which, depending on the species, is reached by 5 to 9 days after fertilization. After collection, embryos are placed into a hypertonic solution containing a CPA, such as glycerol or ethylene glycol (see Leibo, 1992). These agents act to mildly dehydrate the embryo before and during the cooling process. Today, most farm animal embryos are frozen in 0.25-ml or 0.50-ml plastic straws, like those used for freezing bull semen.

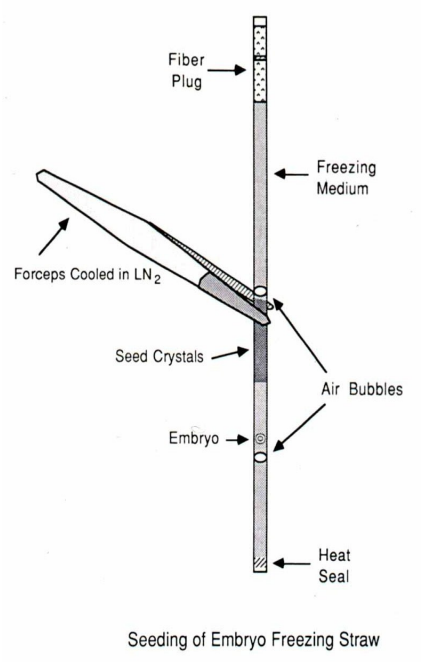
Because embryos are a collection of many interacting individual cells, whereas each sperm consists of a single cell, the freezing protocols for embryos are generally more sophisticated than those used to cryopreserve semen. For more details on the procedures for cryopreservation of farm animal embryos see Appendix G. Cellular properties often vary between species and between the stages of embryonic development. This requires the cryopreservation procedure to be adjusted to the species to minimize damage to the embryo and optimize survival rates (Rall *et al.,* 2000) (see Chapter 7 for basic principles of cyropreservation) The most often used embryo freezing method is the slow freezing technique, which is based upon a reversible dehydration of the cells that prevents the most damaging effects of intracellular ice crystallization. Most of the technicians that use the slow freezing technique employ an automated embryo freezing machine. These machines can be adapted to work under field conditions.

After the embryo and cryoprotectant are placed in the plastic straw, one critical step in the freezing process is "seeding", which is the act of purposefully inducing ice crystal formation in the cryoprotectant solution surrounding the embryo (Figure 8.2). After embryos are cooled to approximately -35°C, they are plunged into LN_2 for storage at -196ºC. A summary of the methods and their applications is presented in Leibo (1992) and Rall (1992).

Several factors have been shown to be critical in determining the success or failure of cryopreservation: 1) the embryo quality - as estimated from morphology following examination with a stereo-microscope; 2) the time from embryo collection to the onset of freezing, which should be no longer than 3 to 4 hours; and 3) the appropriateness of freezing and thawing procedure for the type of animal embryos being cryopreserved. Field reports indicate that *Bos inducus* cattle embryos do not survive the freezing process as well as with *Bos taurus* embryos. The greater lipid content found in most *Bos inducus* embryos at freezing may explain the decreased post-thaw survival rates (see Ballard *et al.,* 2007; Looney *et al.,* 2008).

[17] http://www.fao.org/DOCREP/003/X6525E/X6525E00.HTM

Figure 8.2. Using a forceps to induce ice crystal formation (seeding) in the plastic straw (drawing from R.A. Godke).



8.2.6. Cryopreservation of embryos using vitrification

As explained in Chapter 7, vitrification is a process which uses the rapid increase in the viscosity of solutions during freezing to obtain a glassy solid phase both inside and outside the cells, without formation of ice crystals (see Rall & Fahy, 1985; Rall, 1992). Vitrification is a more rapid procedure that uses a high concentration mixture of CPA. Embryos placed into vitrification solutions are plunged directly into LN2, saving valuable time and eliminating the need to purchase an embryo freezing machine (Vajta and Kuwayama, 2006; Vajta and Nagy, 2006). For further details on vitrification procedures, see Vajita *et al.,* (2005) and Vajta and Kuwayama (2006). Although vitrification is a quick procedure and does not require special equipment, it can be technically more demanding and typically yields pregnancy rates that are 10 to 15% less than the slow freezing technique until one has gained enough experience to have mastered the technique.

Success rates with vitrification in cattle are now approaching the rates with conventional embryo freezing (Seidel and Walker, 2006). Commercial kits for vitrification of livestock embryos are now available. As vitrification methodologies improve, there are some indications that the approach may have some advantages over standard slow freezing procedures in cattle (Vajita *et al.,* 1997; Visintin *et al.,* 2002). The future for vitrification technology appears promising, especially for animal embryos that have lower viability following conventional cryopreservation, such as pig embryos and embryos produced via IVF. At present, good success is being reported using vitrification to cryopreserve horse oocytes.

8.2.7. Embryo sexing and genetic diagnosis technology

Sexing of embryos and selection prior to cryoconservation may decrease the costs of storage and particularly of subsequent thawing, transfer, and production of offspring, especially if a greater proportion of animals of a particular sex are desired in the future.

One simple approach to gender determination is to bisect the embryo and identify the sex of one half of the embryo. Once the sex is established, then the remaining half of the embryo is transferred to a recipient female (e.g. Nakagowa *et al.,* 1985; Herr & Reed 1991). Using another approach, White *et al.,* (1987) bisected bovine embryos and sexed one of the half-embryos by using an H-Y antibody procedure. Then each of half-embryos of the pair was transferred to a

different recipient animal. The success rate for embryo sexing was 90%, and there was no significant difference found in pregnancy rates between the sexed half-embryos and control half-embryos (47% vs. 44%).

Studies using polymerase chain reaction (PCR) technology on fresh and frozen-thawed animal embryos clearly indicate that embryo biopsy techniques can be used as a tool for embryo sexing (Peura *et al.,* 2001; Kirkpatrick & Monson, 1993), without reducing post-biopsy transfer pregnancy rates. With today's embryo sexing technology, only a few cells from the trophoblast of the embryo are needed to conduct the *in vitro* procedures. In fact, the equipment and the supplies needed to sex bovine embryos are commercially available for use by veterinarians and livestock producers worldwide. If the instructions of these commercial embryo sexing kits are carefully executed, the success rates have been reported to approach 100% for cattle embryos.

At present, research efforts are directed toward minimally invasive embryo biopsy approaches to harvest cells to be used for the identification of potential genetic abnormalities and diseases prior to transferring the embryo. In the near future, cells from embryonic biopsy will be used by breeders and AI companies to identify genotypic and/or phenotypic traits of the embryo by using quantitative trait loci (QTL) and genomic selection technology. The potential for use of genomic information to select the appropriate embryo to transfer would be of marked benefit to the commercial breeders. Various companies have recently started making genome-based technology available on a commercial basis to livestock producers. Although gene banking for breed reconstitution typically aims to preserve as much genetic material as possible, technologies for genomic selection of embryos will be particularly useful for cryobanking with the objective of gene introgression (See Section 6.3). For general cryoconservation programmes, these technologies may also be useful for selecting animals or embryos that will conserve the most genetic variability in the gene bank.

8.3. Oocytes

8.3.1. Conventional oocyte collection

Slaughterhouse ovaries are often used to harvest oocytes for research purposes and can be an option for cryoconservation, especially if the genetic characteristics of the donor animals (i.e. beyond breed) are not important and the germplasm is expected to remain within the country and strict adherence to OIE sanitary standards for export is not necessary. The ovaries of the donor females are collected immediately after slaughter, placed in reclosable plastic zip-lock bags and kept warm en route to the laboratory facilities. In general, one should not cool down the ovaries of farm animals or the embryo production success rate from IVF will be dramatically reduced. Individual bovine oocytes are generally aspirated from small, medium and large-size follicles (see Appendix H), subsequently matured, fertilized, cultured and transferred either fresh to a recipient, or frozen. Usually, 4 to 12 oocytes per ovary can be harvested (using a sterile needle and plastic syringe) from cattle ovaries. Once collected, the oocytes are evaluated for quality and placed in oocyte maturation medium overnight (e.g., 20 to 23 hours for cattle oocytes) in preparation for *in vitro* maturation (IVM) and IVF procedures (see examples in Appendix I and J). This methodology can be used to collect oocytes from nearly all species of livestock and is relatively simple, inexpensive, and highly recommended for training students and laboratory personnel.

This procedure could produce offspring from unforeseen female casualties, old or clinically subfertile females. Using a novel conservation strategy, one might systematically recover the ovaries from all females of an endangered breed that die or go for slaughter. These oocytes could then be fertilized in the laboratory and the subsequent embryos frozen for transfer to recipient females at a later date. In the case of compulsory termination of a herd due to a nonviral epizootic disease outbreak, it is still possible to produce clean embryos (using the IETS and OIE animal health and embryo handling procedures) for disease-free transfers to subsequently re-establish the initial animal herd.

8.3.2. Transvaginal ultrasound-guided oocyte collection (TUGA)

Oocytes may also be collected from live donors via follicle aspiration by using one of three basic surgical procedures that have been reported over the years. The first is the standard laparotomy technique to expose the ovaries, which can be executed using various anatomical approaches on all the large farm animals. Although this method has been successful in cattle and horse, today it is most often used in sheep, goats and pigs. The second is the endoscopic approach, which is also most commonly in sheep and goats, but has also been used for follicle aspiration in cattle and horses. The third approach uses transvaginal ultrasound-guided oocyte recovery (TUGA) that is now most commonly used in cattle, buffalo and horses. TUGA is often referred to by its common name, "ovum pick-up (OVU). All three of these oocyte collection approaches require the services of a skilled technician; a descriptive overview of the TUGA procedure is presented in these guidelines.

TUGA was originally developed in humans to retrieve oocytes by using ultrasonography to evaluate the ovary and to transvaginally guide a hollow needle into to each visible ovarian follicle (e.g. Wikland and Hamburger, 1984; Dellenbach *et al.,* 1985). The needle is attached to a source of vacuum and is used to aspirate fresh *in vivo* oocytes from the follicle, which are then subjected to IVM, IVF and then *in vitro* culture procedures. The procedures for humans were then modified to harvest oocytes from live cattle (e.g. Callesen *et al.,* 1987; Pieterse *et al.,* 1988, 1991) and other species. TUGA is now routinely used in cows, goats, mares and more recently in pigs and exotic large, hoofed species.

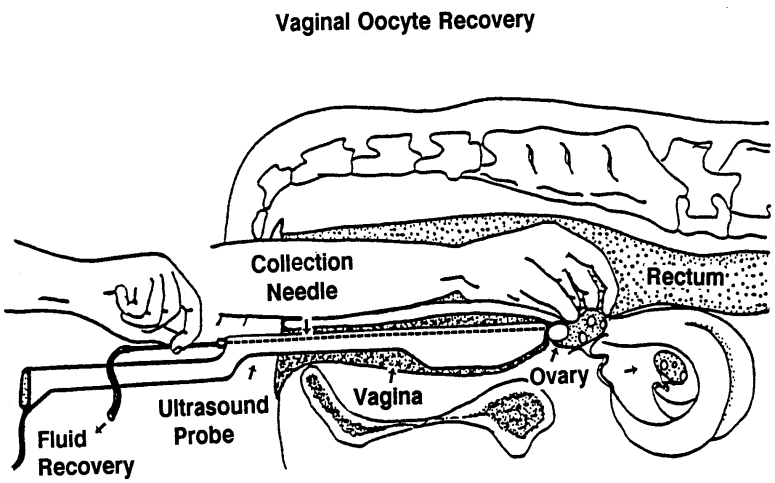
TUGA can expand the time that animals can be reproductively active. For example, both pregnant cows and mares continue follicle wave development during early to mid-gestation. A novel approach is to take advantage of these developing ovarian follicles to produce IVF offspring from oocytes during the early stages of pregnancy. This procedure does not compromise the pregnancy and oocyte yield actually tends to be greater than from non-pregnant animals (Meintjes *et al.,* 1995b; Cochran *et al.,* 1998a,b). This procedure can be especially useful for larger farm animals, because they tend to have only one offspring per pregnancy and their relatively long gestation periods mean that potential donors pass through long periods when they cannot be used for embryo production. In addition, TUGA can be used on animals before they reach sexual maturity. Oocytes from prepubertal sheep and cattle have produced IVF offspring (see Looney *et al.,* 1995; Bols *et al.,* 1999). Oocytes from near-term bovine fetuses and new born calves are being harvested from farm animals for IVF procedures but offspring have not been born at the time of this publication.

To retrieve the oocytes for IVF, a trained professional inserts an ultrasound-guided stainless steel needle through the wall of the vagina near the cervix to extract the oocytes from the follicles visible on the ovaries. The procedure is conducted on the small, medium and large-size follicles on both ovaries of the donor female.

8.3.2.1. TUGA in cattle

In cattle, the donor female is restrained in a suitable holding chute and administered an epidural block. A convex ultrasound 5-megahertz (MHz) sector transducer is fitted onto the distal end of a specially-designed plastic handle to visualize the ovaries on the screen of the ultrasound monitor. The plastic handle (with a latex protective covering) is inserted into the vaginal canal, and then the ovary is grasped *per rectum* and placed against the transducer in the vagina (Figure 8.3).

Figure 8.3. Transvaginal ultrasound-guided oocyte aspiration (TUGA) in the cow (Courtesy of Dr. A. Meinjtes).



Follicles are identified as black (hypoechoic) circular shapes on the monitor screen. An 18-gauge, 55- or 60-cm long needle is inserted through the needle guide in the plastic handle. This needle is connected to a suction pump by means of polyethylene tubing, passing through an embryo filter or into a 50-ml conical-shaped test tube for collection of the follicular fluid containing the oocytes. The basic solution used for this procedure is PBS with 10% bovine serum, antibiotics and heparin added to this medium. Using this aspiration method, 60 to 70% of the medium to large-size follicles punctured result in oocytes recovered, with an average of 3 to10 oocytes per nonstimulated donor female. A significant training period is required to become proficient using this oocyte collection procedure in cattle.

Aspirations are usually performed once-a-week, but have been performed twice-a-week for up to 3 months in cows (Gibbons *et al.,* 1994; Broadbent *et al.,* 1997) with no overt effects reported for the donor females. With TUGA and IVF, the potential exists for more embryos to be produced in a shorter period of time than with conventional ET, because the procedure can be realistically repeated on the same cow 3 to 4 times a month. In addition, using TUGA for oocyte collection does not require any hormone treatment of the donor. The frequency of recovery can be much greater than for embryo collection after superovulation (up to 80 recoveries during one year in cattle compared with no more than six collections when embryos are collected nonsurgically). Oocytes can be harvested from donor cows at anytime of the estrous cycle including at standing estrus and the growth phase of the first follicular wave (Paul *et al.,* 1996). Although superovulation is not necessary, the number of oocytes collected per female can be increased by treating the female with gonadotropic hormones prior to the aspiration procedure. IVP of embryos generally results in 1 to 3 bovine embryos for transfer per oocyte collection procedure for nonstimulated donors.

8.3.2.2. TUGA in buffalo

Similar oocyte collection procedures to those used in domestic cattle are now being developed for buffaloes in various parts of the world. Again, the primary objective is to subject the oocytes collected to IVF procedures for fresh transfers or for cryopreservation. Using TUGA for successful harvesting of oocytes has been reported for the Swamp buffalo (Pavasuthipaisit *et al.,* 1995; Techakumphu *et al.,* 2004; Promdireg *et al.,* 2005) the Italian Mediterranean buffalo (Boni *et al.,* 1996) and the Murrah buffalo (Gupta *et al.,* 2006). Presently, the basic bovine IVF procedures are being fine-tuned to use buffalo oocytes for embryo production.

8.3.2.3. TUGA in the horse

The horse has presented a unique problem for researchers working in the assisted reproductive technology area. As noted previously, although embryo collection and transfer are relatively simple in the mare, superovulation is generally ineffective. Due to the unique anatomical structure of the horse ovary, only one ovum usually ovulates at the appropriate time during each estrous cycle. Also, for some yet unknown reason, typical IVF procedures have not worked consistently in the horse.

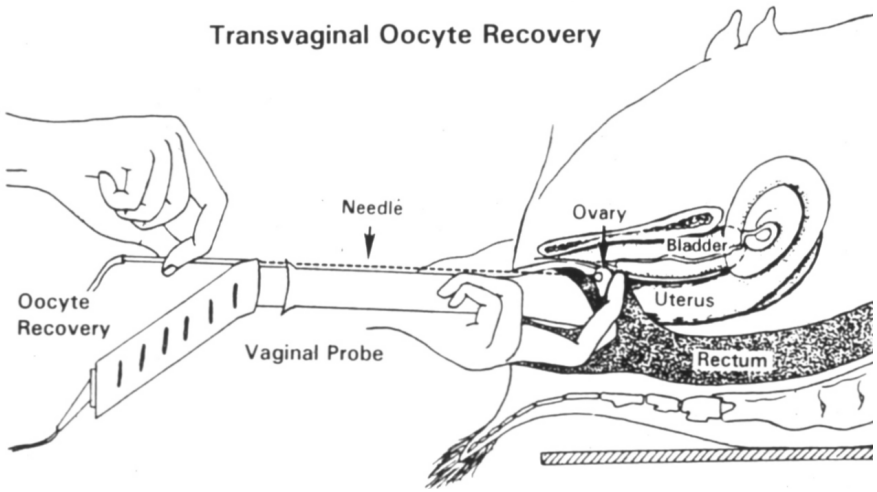
Even though only one follicle normally matures and ovulates during an estrous cycle, mares also are thought to have one or two waves of multiple follicles during that estrous cycle. Once again, this developing follicle population makes it possible to use TUGA to collect oocytes from live mares (see Brück *et al.,* 1992; Cook *et al.,* 1992) for the production of embryos for transfer. The first foals produced from aspiration of live mares were produced by using ICSI (See Section 4.7.2.). After ICSI, embryos are surgically transferred at the 2- to 4-cell stages into the oviducts of suitable recipients, since the culture of IVF-derived equine embryos has still not been perfected.

The aspiration setup for mares is similar to that used in cattle, but with some modifications. Briefly, mares require sedation instead of an epidural block, and a 12-gauge needle is used to puncture the follicles. Extra rinsing of the follicle is necessary in the horse, since the oocyte is usually well-embedded in the follicle wall. In this case, the needle recommended is a double lumen needle, so that the follicular fluid can be aspirated, and the medium used to again rinse the follicle (two to four times per follicle). The follicular fluid is collected into a 500-ml bottle, and then later passed through the standard embryo filter. Using this modified method, oocytes have been successfully recovered from mixed-breed cyclic mares and ponies (Meintjes *et al.,* 1995a), pregnant mares (Meintjes *et al.,* 1994, 1996) and free ranging zebras in South Africa (Meintjes *et al.,* 1997). Oocyte recovery rate usually ranges between 50 and 75% of follicles punctured per mare. After attempting IVF or the sperm injection procedures, developing 2- to 4-cell stage embryos are transferred surgically into the oviducts of recipient mares. The ICSI procedure (Section 4.7.2.) appears to be the method of choice at this point to produce horse embryos in the laboratory.

8.3.2.4. TUGA in small ruminants

Goats are another farm animal species in which IVP has proven successful. Transvaginal aspirations have also been performed on cyclic and noncyclic adult goats with good success (Graff *et al.,* 1999). Although the oocyte recovery rates usually range from 60 to 80% for the follicles punctured per donor female, there are some problems with aspiration of ovarian follicles from does using TUGA. First, the ovaries cannot be grasped *per rectum* for optimum visualization with ultrasonography. Secondly, since the ovaries cannot be easily grasped, it is more difficult to puncture follicles and aspirate the oocytes. Although the methodology for puncturing the follicle is similar to the cow and the horse, the goat must be sedated, put under anesthesia and then placed in dorsal recumbency for the procedure (Figure 8.4).

Figure 8.4. Ultrasound-guided oocyte aspiration in goats (Graff *et al.,* 1999).



Manual pressure is placed on the abdomen in an effort to stabilize the ovaries for aspiration. The ultrasound probe, which is smaller than that used for cattle, buffaloes and horses, is inserted into the vagina with the convex transducer at the distal end of the handle. The aspiration proceeds with no need for extra rinsing of the follicles to recover the oocytes.

Oocyte recovery is usually a little slower than desired because not all follicles can be visualized, and not all follicles visualized can be adequately punctured due to the difficulty of securing the ovary. Frozen-thawed goat embryo offspring have been produced using the transvaginal aspiration procedure together with IVF methods (Han *et al.,* 2001). Although this non-invasive procedure requires expertise and patience, it is an important assisted reproductive technology that can decrease the risk of ovarian adhesions or death from using the standard surgical method to harvest oocytes from valuable donor goats. An efficient TUGA method for harvesting oocytes has not as of yet been developed for sheep, however.

8.3.2.5. TUGA in other species

TUGA has also been used successfully in other animals, with modifications made primarily to account for anatomical differences among species. For example, TUGA been used successfully in adult pigs (Bellow *et al.,* 2001), llamas (Brogliatti *et al.,* 2000) and various exotic hoofstock, such as the Red deer (Berg *et al.,* 2000), the Sika deer (Locatelli *et al.,* 2006), the rare Bongo antelope (Pope *et al.,* 1998; Wirtu *et al.,* 2009) and the African eland (Wirtu *et al.,* 2009). Therefore, this technique may be particularly useful for managers of gene banks that are a cooperative effort between agricultural and natural resources ministries to preserve domestic and wild animal genetic resources.

8.4. Somatic cells

As mentioned previously, collection of tissues other than germ cells and embryos can be useful for gene banking, either for the production of new animals (through SCNT) or to obtain genetic and health information about the animals sampled (DNA isolated from cells).

8.4.1. Tissue

Somatic cells for subsequent use in DNA analyses or SCNT may be sampled from the tissue of live animals or from animals shortly after death (Silvestre *et al.,* 2004). Because the requirements for the two objectives (DNA or SCNT) differ, separate protocols have to be used for each. For DNA, one approach is to use a sterile scalpel blade to aseptically remove thin strips of skin from the body surface (e.g., shoulder area) of an animal and then place them in a pre-labelled sterile screw-top vial for transport to the processing laboratory. Collection can also be done easily with a sterile hole punch made in the peripheral border of the ear of the live animal or at the time of death. Prior to freezing (or vitrification), the tissue samples should be wrapped in blotting paper

moistened with PBS and maintained at 4ºC to minimize degradation of the samples. As an alternative to cryopreservation, tissue samples can be preserved through dehydration by storage of each sample in a glass vial containing a grain of silica gel. In addition, for DNA analyses in the relatively short-term (< 2 years) hair samples may be an option. Hairs must be plucked from the roots and then dessicated, placed in separate labelled containers for each animal and stored in a dry environment. For extraction of DNA, various protocols exist, but the simplest approach is to use a commercial kit designed for the specific tissue.

Collection of tissue for SCNT needs to follow a more stringent protocol than for samples intended for DNA analyses. Appendix K presents two protocols, the first for simple and low-cost field collection and freezing (Groenveld *et al.,* 2008) and the second for the situation where samples will be processed in the laboratory. In both cases the same procedures are generally applicable to all species, particularly to mammalian species.

8.4.2. Blood

Blood samples collected from live animals or from animals shortly after death may be used as for DNA analyses or cloning via SCNT. The DNA from the blood of mammals comes from the white cells only, because the red cells do not have nuclei. Birds have DNA in both red and white cells thus, a smaller blood volume is required from birds to obtain ampoule quantities of DNA for analyses. Blood is relatively simple to obtain in quantities of DNA that are sufficient for genetic analyses. Collection of blood from the jugular or caudal vein with a needle and vacutainer tube is the common procedure in mammals, whereas poultry are usually sampled from veins in the wing.

Collection of two vials of whole blood (total of 10 to 14 ml) is recommended at the time of collection any other germplasm for animals selected for the gene bank to reduce the risk of the accidental loss of all the sample from an individual. White blood cells can be harvested from fresh whole blood following centrifugation. The buffy coat is carefully pipetted from the sample and divided into at least two small pre-labelled sterile vials for use in nuclear transfer. The vials are then frozen in nitrogen vapour and stored in LN2.

If only DNA is needed, it can be extracted using commercial kits and following their specific procedures. For long-term storage, the extracted DNA should be put into labelled aliquots of 50 µl, with a concentration of 200 µg/ml. Division into aliquots will provide repeated access while avoiding freezing and thawing of the entire sample. The DNA may be safely stored at 4°C for 2 months before dividing into aliquots, provided the preparation is pure enough, otherwise rapid transfer to long-term storage is necessary. For long-term storage, DNA can be placed maintained at -20°C or lower (-80°C or in LN2) but the latter is not necessary

8.4.3. Gonadal tissue (poultry)

As noted in Section 4.7.1, recent studies have reported that ovarian tissue can be harvested from female immature chicks, frozen, thawed and transferred back to other young females (see Song and Silversides, 2006). Subsequently, newly hatched chick testicular tissue has been harvested and transplanted successfully to host chicks, resulting in live offspring born from sperm derived from the donor testicular tissue (see Song and Silversides, 2007). The procedures used for cryopreservation of ovarian and gonadal tissue are given in Appendix L.

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9. SANITARY RECOMMENDATIONS

Each country will need to balance their needs to preserve breeds of interest and compliance with national and international health regulations. This decision will be based in part on the types of existing diseases and how contagious, virulent and damaging they are to animal production. Certainly, animals exhibiting a highly contagious and possibly fatal disease like Foot and Mouth should not be collected except in the most dire of circumstances (i.e. if no other non-infected animals exist).

Diseases of concern are country dependent, as are the health regulations a gene bank should follow in developing collections. However, if the gene bank is interested in distributing germplasm to another country, especially those with widely divergent health concerns, attention to the OIE health regulations will be needed (www.oie.org). International transfer of germplasm that does not conform to these regulations could put a country at risk of losing its OIE export status.

The primary issue for a gene bank collecting germplasm in the field is to minimize the risk of spreading diseases from farm to farm while collecting germplasm from animals belonging to different owners. Additionally, efforts must be made to reduce the risk of disease spread during the utilization of germplasm that the repository has collected and cryopreserved.

Achieving a high level of both health testing and sample preparation is facilitated when germplasm is collected in an AI centre or other controlled environment. Maintaining these protocols becomes more problematic when field collections are performed. In certain instances it may be necessary to obtain a waiver from national or international veterinary agencies so that endangered breeds can be collected and cryopreserved. Obviously, in field collection situations maintaining animals in quarantine for any length of time may be impractical or even impossible. In the best case scenario, such actions would increase collection costs. A potential solution to help address the issue of possibly collecting germplasm from infected animals in the field is to draw blood samples from donor animals and have the blood sample tested for the disease(s) of interest.

Excellent reference material concerning regulations necessary for the collection, testing and processing of germplasm include:

- Regulations necessary for the collection, testing and processing germplasm samples for international exchange (OIE website: www.oie.int),
- AI centre protocols of the National Association of Animal Breeders (www.naab-css.org),
- EU-regulations for AI centres and international exchange of germplasm (http://ec.europa.eu/food/animal/index_en.htm).

Although the above noted documentation may not directly apply to all countries, application of the procedures described will increase the level of sanitary safety and thus decrease the possibility of disease transmission.

9.1. Collection and processing facility

It is likely that the animals brought into a collection centre will be present for only a relatively short period of time. Therefore, cost effectiveness of quarantine and health testing is a consideration where an optimal solution is needed. Three components of a collection facility have to be considered in minimizing the risk of disease transmission.

1. As animals enter the collection facility they should be maintained in isolation barns or pens for a specified period of time during which they can be tested for various diseases of concern, placed on selected rations, and trained for collection. Ideally, personnel that handle quarantined animals will not be involved in the care or collection of animals that have already been tested. As previously noted in Chapter 5, for ease of management the

quarantined area should utilize an all-in all-out policy, where animals not meeting the predetermined health criteria force all animals to not be accepted.

2. Once animals have passed the health tests they are moved into the main facility for animal maintenance and collection. At this stage in the collection process the primary health concerns arise from the potential for introducing a disease from outside the facility. Such risks can be minimized by requiring animal handlers to follow specific sanitation protocols and by eliminating the presence/exposure of the area to rodents or wild birds. The other potential risk, contamination from bedding, can be addressed by cleaning animals prior to collection. Even though animals in the collection facility have passed through quarantine, equipment for germplasm collection such as AV and collection tubes should be kept clean and changed for each animal.
3. After collection, samples of semen and other genetic materials are transferred to a laboratory. Protocols for Biological Safety of at least Level 2 (e.g. www.cdc.gov) need to be in place in order to maintain minimal sanitation standards and prevent any cross-contamination between samples.

9.2. Field collection

Although germplasm collection in a closed facility is desired for control of health and sanitation, in some instances it may be more practical and cost effective or even necessary for gene banks to perform collections in the field. During field collection, the sanitation of each location must be respected to prevent the spread of sickness or disease by the collectors. When samples are to be received at the gene bank from a collection site the shipping boxes and any supplies associated with that location are never to be used at other locations. The boxes and supplies may be reused in the future if they have been sanitized, but they should only be used at the same collection site. If frozen materials are arriving at the gene bank then the LN2 tank or dry shipper should be sanitized with a 10% bleach solution after it has been warmed to room temperature.

In the event that personnel are travelling from collection site to collection site, then very specific sanitation practices should be considered. The undercarriage and tires of the vehicle should be washed, preferably with a disinfectant, after visiting each location. Likewise, the boots of the personnel should be disinfected or covered by using disposable boot covers that are then discarded after leaving each location. Clothing should also be laundered or changed between collection sites or disposable suits should be utilized. Poly vinyl or nitrile gloves should be worn and changed between handling different animals.

When using equipment such as syringes and needles, the articles are never to be shared between animals and they are to be disposed of properly according to local regulations following use. If the equipment is not disposable in nature, such as an electro-ejaculator, it must be sanitized and rinsed between animals in order to avoid spreading pathogens and potential illness.

9.3. Disease testing

As mentioned previously, germplasm should not be collected from animals that are clearly infected with a highly contagious disease if at all possible. In general, sampling of only healthy animals is preferable, regardless of the disease. Hence, the quarantine of animals is recommended for animals entering a collection centre. Infected animals may not show any outward symptoms of disease, even if they spend a certain time in quarantine. For this reason, it's recommended to collect samples of blood or other tissues (e.g. nasal smears) for more comprehensive tests for the presence of disease agents.

9.4. Storing samples

9.4.1. Prevention of contamination during storage

Cross contamination of samples in LN2 is possible, but this has only been demonstrated to be of consequence in studies where the contaminant was placed in the tank (Grout & Morris, 2009). Otherwise the probability of this happening seems to be very low. Nevertheless, because

pathogen transmission is possible, cautionary practices should be undertaken to minimize any potential for cross contamination. This practice begins by determining how the material will be handled and stored. For example, semen straws can be sealed with a variety of substances including Poly Vinyl Alcohol (PVA) powder, clay, metal ball bearings, heat sealing or other similar methods. Whereas the PVA powder, clay and ball bearings are inexpensive, the quality of the seal is inferior to that achieved by heat sealing and consequently the potential for contamination through leakage or rupturing of straws is greater.

9.4.2. Dealing with samples of questionable sanitary status

If the sanitary level of previously stored samples is questionable, then the semen straws or other storage devices can be cleaned with ethanol and allowed to dry following thawing. However, a more important question may be how to deal with newly collected and frozen samples from sources differing in biosecurity, such as from collection in the field rather than from an approved collection centre. In such an instance, separate storage of samples of uncertain sanitary status should also be considered. For maximum security, distinct storage sites can be used and at the minimum, samples of unknown status should be stored in separate tanks. In addition, specific LN2 tanks designated for quarantine purposes should be made available. Then, for example, samples that are received from places other than designated collection facilities or AI centres (i.e. facilities that are known to be sanitary) can easily be segregated and transferred, if desired, so that clarity about sanitation status is determined. For example, if samples collected in the field by the gene bank laboratory personnel are considered suspect for sanitation and health issues, then they can be quarantined until results of blood tests from the sampled animals are returned demonstrating a clean bill of health or stored in separate tanks. According to the country, germplasm from different species may have to be stored separately, regardless of the health status and site of collection.

Considerations regarding segregation of germplasm based on knowledge of health and sanitary status are of particular importance when germplasm may be transferred internationally and adherence to OIE standards is critical. Nevertheless, given the potentially high value of stored germplasm and the fact that the stored samples may in the future be the only means by which to recover a lost breed or support the maintenance of a highly endangered breed, the potential risks of joint storage of samples with known and unknown levels of sanitation must be given serious consideration.

Regardless of the system used, sanitation status of a given sample is key information to be included in any gene bank data base and information system (See Chapter 10). In this way, all samples can be tracked and managed properly regardless of their storage site.

Reference

Grout, B.W. & Morris, G.J. 2009. Contaminated liquid nitrogen vapour as a risk factor in pathogen transfer. *Theriogenology,* 71: 1079-1082.

10. DATABASE AND DOCUMENTATION

Proper and accessible documentation is vital for the future use of any stored gene bank material. A primary focus in the documentation of samples in a gene bank is development, implementation and utilization of a database. A database is essential because it is the management system that will catalogue, summarize, query and retrieve information required to establish and operate the gene bank. Basic information about gene bank collections should be easily accessible without the need for any additional information from outside the database. Gene bank management relies on the database to manage routine gene bank operations (e.g. quality control testing, sample identification, sample location, current inventory, movement among collections and de-accession) and to support management decisions.

The database serves as the primary conduit for receiving information about samples in the collection. The outflow of information is just as essential as the input of information so that potential requestors have a way to view the contents of the collection and make choices about their request. To insure the broadest access to the information contained in the database, it needs to be linked to the internet. Addition of internet access facilitates awareness about the country's AnGR programme and makes it easier for the diverse users to access information and make use of the stored germplasm.

Databases for gene bank management can be very diverse. For example, the most basic information storage could be developed and used via a spreadsheet programme like Excel. More complicated databases can be developed by using computer software specifically designed for database construction. With such software, a broad array of databases, differing vastly in complexity, can be developed.

From the onset of initial gene bank planning, the database must be recognized as being of central importance in the establishment of the gene bank, both in terms of day-to-day management but also for allowing potential users of the gene bank access up-to-date information regarding the material that is contained in the collection.

10.1. Components of a gene bank information system

All databases have a tabular structure that has either a one-to-one or one-to-many relationship among the tables in the database. Initial design of the database starts with the close cooperation between gene bank managers, database developers/operators and potential user groups. This step serves to ensure that the needs by all users for information can be met. Once information needs have been identified, usually a graphic scheme will be developed that illustrates the various relationships that exist within the database. Whereas development of the database itself is a significant and essential task, just as important are the additional elements necessary to input and extract data from the database. The following is a list of such components:

- Data input screens – facilitate data input and mimic data collection forms;
- Data edit screens – have the capability to make changes to data elements plus some automated features that allow changes to more than one record at a time;
- Records review capability – facilitates the recall of individual or group records;
- Data summary – summarizes various elements in the database and calculates statistics that may be of interest to users or managers;
- Query capability – the ability to extract specific pieces of information from the database;
- Information output method – users need options on how requested data is presented in tabular or graphical form as well as the type of file to which the requested data can be exported;
- Data entry and edit control – database managers need to have control over who can enter and edit data in the database. Usually this is accomplished by making access to entry and edit functions password protected; and
- Inter-operability – there be a need or desire to link to and exchange information between the gene bank database and databases operated by other national or international agencies.
- Access via internet - In addition to the items listed, serious consideration must be given by gene bank managers, database users and database staff as to what extent information will be available on the internet. There may be a need to not make some information publically available, such as where certain samples are stored in the repository. This information may thus be password-restricted or subject to some other form of access control. In general, outside users will be granted "read-only" status for the database.

As part of the database construction decision, gene bank managers should be aware that several options and opportunities exist to utilize database systems have already been developed or are in production by various gene banking groups. Database development requires specific expertise that may not be available in all institutions or countries. Therefore, the use of existing databases and software packages or joint development of a database across countries may be a preferred option for gene bank managers. In addition, use of an existing database application will usually facilitate inter-operability. Among the countries and regions that have already developed databases for documentation of cryopreserved material are the following:

1. Supported by the European Commission, the EFABISnet project developed the CryoWEB database tool. This tool is already implemented in a number of European countries and is also integrated with the EFABIS breed database. The CryoWEB database software is available under a free GPL license and can serve as a basis for further adaptation or development (see: http://cryoweb.tzv.fal.de/).
2. The National Animal Germplasm Programme in the USA, EMBRAPA of Brazil, and Agriculture and Agri-Foods Canada have joined together and developed an internet based database for management of germplasm collections that also offers the option of performing cross country comparison of germplasm collections. (See http://www.ars.usda.gov/Main/docs.htm?docid=16979).

10.2. Database information set

In developing descriptors for the database, each country has to determine what information it wants to maintain and what is needed to thoroughly describe the samples maintained in the repository. Tables 10.1 and 10.2 provide a list of minimum and recommended information fields that should be collected for every donor animal and sample in the gene bank.

Obtaining these descriptors may be difficult and in some situations some of them may not exist. A potential solution for germplasm collectors is to obtain this information while performing field collections. Another approach is to develop survey sheets containing questions about the needed information. Germplasm collectors can interview the owner to solicit the needed information or give the livestock owner the sheet to complete and return.

Table 10.1 Donor animal information: recommended minimum and additional database fields.

Trait	Type	Minimum	Recommended	Comment
Animal ID				
Owner ID	Alpha-numeric	X		
Repository ID	Alpha-numeric	X		
Association ID	Alpha-numeric		X	
Markings			X	e.g., tattoo
Animal birth date	Alpha-numeric		X	
Sex	Alpha-numeric	X		
Source				
Breeder Name	Alpha		X	Contract with original owner (if any) attached to the database
Owner Name	Alpha	X		
Geographic location (GIS coordinates or mailing address)	Alpha-numeric	X		
Taxonomy				
Species	Alpha	X		
Breed	Alpha	X		
Population	Alpha	X		
Environment	Alpha		X	Arid, Semi-arid, Temperate, Sub-tropical, Tropical
Management System	Alpha		X	Extensive, mixed crop-livestock, industrial
Phenotypic Measures				
Body weights	Numeric		X	Birth weight, weaning weight, mature weight
Visual identifiers	Alpha-numeric		X	Color, horns, photograph
Production measures	Alpha-numeric		X	Milk yield, fleece weight, litter size, etc.
Genetic Measures				
Pedigree	Alpha-numeric		X	3 generations if possible
Genetic test results	Alpha-numeric		X	e.g., Halothane, BLAD, scrapie
Genetic markers	Alpha-numeric		X	Microsatellite, SNP
Breeding values	Numeric		X	e.g., production traits
Breed Information				
Census data	Numeric		X	
Phenotypic descriptors	Alpha-numeric		X	Average weights
Genotypic descriptors	Alpha-numeric		X	Know genetic attributes
Production systems	Alpha-numeric		X	Production systems where the breed is prevalent

Table 10.2. Sample information: recommended minimum and additional database fields to be associated with animal ID.

Trait	Type	Minimum	Recommended	Comment
Collection				
Date				
Location				
Sample quality				
Semen				
- Temperature at arrival in lab	Numeric	X		
- pH at arrival in lab	Numeric	X		
- Pre-freeze motility	Numeric		X	
- Post-thaw motility	Numeric	X		
Embryo				
Grade before freezing				
Stage of development				
Quality after freezing				
Straw Information				
ID	Alpha-numeric	X		
Freeze date	Numeric	X		
Species	Alpha-numeric	X		
Breed	Alpha-numeric	X		
Storage Information				
Kind of straw or pellet or else	Alpha-numeric	X		
Tank	Numeric	X		
Placement in tank	Numeric	X		
Collection method				
Semen	Alpha-numeric	X		
Embryo	Alpha-numeric	X		
Oocyte	Alpha-numeric	X		
Somatic cells	Alpha-numeric	X		
Freezing protocol used[1]				
Semen	Alpha-numeric	X		Detailed protocol attached to the database
Embryo	Alpha-numeric	X		
Oocyte	Alpha-numeric	X		
Somatic cells	Alpha-numeric	X		
Sample ownership				
Semen	Alpha-numeric	X		
Embryo	Alpha-numeric	X		
Oocyte	Alpha-numeric	X		
Somatic cells	Alpha-numeric	X		
Sample sanitary status				
Semen	Alpha-numeric	X		Details of diagnostic tests attached to the database
Embryo	Alpha-numeric	X		
Oocytes	Alpha-numeric	X		
Somatic cells	Alpha-numeric	X		

[1]Thawing instructions may be included as additional information.

11. LEGAL ISSUES: CONTRACTS AND ACCESS

In the development of country based gene banks there may be need for various types of agreements to acquire germplasm or tissue and to disperse this material when it is requested by potential users. The objectives of these documents are to delineate the rights and responsibilities of the gene bank and users of the gene bank's germplasm/tissue, and, where need be, the donors of the samples. Due to the potential legal ramifications of these transactions the gene bank needs to put in place a clear set of policies that will guide the development of the various types of agreements needed to execute the gene banks mission. For example, in the United States' genetic resource system, the Congress enacted legislation stating that material in the public collection will be distributed to requestors free of charge. With such a policy in place the gene bank has clear guidance on one aspect of germplasm distribution. But as this chapter will point out, this policy only covers one issue of germplasm release. Each country needs to establish a clear set of critieria for all aspects of acquisition, storage and use of gene bank material.

In developing policies and general agreements for acquiring and dispersing germplasm, a suggested guiding principle is that the security of AnGR and enhancement of the country's livestock sector be facilitated. In other words, conditions placed upon acquisition or release of germplasm should not be so restrictive as to put valuable AnGR at risk by impeding the development of germplasm collections or the use of the material stored in the repository.

11.1. Gene bank structure for handling agreements

Gene banks have to contend with a range of different types of agreements concerning the acquisition and release of germplasm. Due to the long term nature of the gene bank's mission and its close relationship with the livestock sector it may be well to establish an advisory committee of interested parties not employed by the gene bank. This committee may or may not be the same as the National Advisory Committee on AnGR described in *Guidelines for the Preparation of National Strategies and Action Plans for Animal Genetic Resources for Food and Agriculture.* The committee's mission would be to provide advice and recommendations on policies for acquiring and distributing germplasm. By doing this, the gene bank will have, through external review, a recommendation on how contracts and other agreements and policies should be structured and thereby garner industry and governmental support.

11.2. Acquiring germplasm

Depending on the country, there may not be specific legislation concerning the exchange of AnGR; rather, the exchange of AnGR may be governed by the broader category of property rights. This situation often exists because historically and individual livestock have been primarily considered private property (of an individual, group of individuals, or company) in most countries. As a result owners have been able to breed and improve their livestock as they have deemed appropriate. Furthermore, livestock owners have generally been free to buy and sell livestock for genetic improvement purposes for centuries (Wood and Orel, 2005). As biotechnologies like AI emerged they have been used as the logical extension for marketing the genetic improvement breeders may have made. To facilitate commercial exchange, buyers and sellers have used a variety of agreements and private contracts. Because livestock breeders own the genetic resource, the gene bank requires a transfer of ownership from the breeder. Alternatively, the breeder may prefer an agreement that facilitates the holding of the germplasm by the gene bank without a transfer of ownership.

As a result of the pre-existing practices for the exchange of AnGR within a country, gene bank managers may likely have to negotiate an arrangement with each owner in order to acquire germplasm samples. Several approaches can be used:

1. The gene bank may buy the animal from the owner, thus obtaining unconditional rights to the AnGR.

2. The livestock owner can donate the sample of germplasm to the gene bank, and by so doing give up all claims to the germplasm donated.
3. The animal owner can charge a fee for access to the animal and the germplasm collected. By doing this the owner may or may not forego further claim(s) on the germplasm collection.
4. The livestock owner could maintain ownership of the germplasm for a specified period of time while it is in the gene bank (also known as an embargo), after which the germplasm becomes the property of the gene bank. Such an approach can protect breeders, for a period of time, from competitors that may want to acquire such samples for the purpose of gaining an advantage. If the owners do not want to forego the rights to the germplasm being stored in the gene bank, managers have to assess if material stored for a long time (and replaced with newer samples) should remain in the gene bank or returned to the owner.

Given the approaches above (particularly 3 and 4), as examples, the gene bank may need to formulate agreements and/or contracts addressing the transfer of the germplasm. The following elements are suggested for incorporation into such a material transfer agreement (MTA).

- Property rights: The ownership to the cryopreserved material should be specified. The rights of the owner (donor) and the gene bank should be defined.
- Costs of collection: Donor and gene bank need to agree about the costs associated with collection, freezing of the germplasm.
- Storage: If there is germplasm which the gene bank regards as important to store but has neither clear ownership nor the potential for ownership, it may wish to arrange for the germplasm owner to pay a storage cost.
- Access: Depending upon the interest of the donor, the agreement may need to stipulate particular conditions for accessing the germplasm (Example 4 above). The simplest approach is to structure the agreement so that any requestor must first obtain permission from the donor before it is released. By doing this, any issues about further use of the material do not involve the gene bank, therefore maintaining its position as a neutral entity. Serious consideration should be given to insure that the release of gene bank material will not harm the competitiveness of the breeder/provider.
- Intellectual property rights: If the gene bank is established as a public good, research results from the use of the material in the gene bank should be publicly accessible without any claims on intellectual property. Such a position will also minimize or eliminate the gene bank's involvement with any type of benefit sharing arrangements.
- Veterinary/sanitary issues: National health policies may play a role in collecting, transferring and using germplasm, thereby creating a need for the gene bank to be cognizant of those conditions in acquiring germplasm. Health status of the donor animal and the cryopreserved genetic material should be defined in the MTA. The MTA should contain a list of diseases for which the animal has been tested at the time of collection.
- Storage sites and quality assurance: A simple statement in any agreement should be made that the gene bank will follow a set of best practices to insure the viability of the samples is maintained.
- Data protection: The provider and gene bank may consider what information they want made publically available about cryopreserved material and the donor.

11.3. Access to the gene bank's collection

As mentioned previously, there are three primary reasons to access stored material:

- national need,
- non-research related breeding of animals, and

- research.

The potential use of the material determines from which collection category the genetic material will be taken.

11.3.1. Requestor's actions

Potential users of gene bank material should initiate the process by submitting a written request outlining their need for the germplasm. The written request should provide the following information about the germplasm needed and its intended use:

- Legal entity and affiliation of the applicant
- Type and quantity of the genetic material requested:
  - species
  - breed
  - number of animals
  - name and registration numbers, if specific animals are requested
  - type of germplasm
  - quantity of germplasm
  - justification for the type of germplasm requested
- Accurate information on the intended use:
  - For breeding purposes – a justification of the need to access stocks from the gene bank. This justification may need to include information on the structure, effective size and performance of existing populations.
  - For access to DNA for research purposes - details about the project including objectives, collaborators and sponsors.
- Types of benefits that could come from obtaining access to the resources.
- The competence of requestor to be able to properly use the genetic material and to maximize success.
- Agreement or waiver by the requestor to accept any risks associated with the health status of the genetic material and to take any subsequent measures necessary to avoid the spread of diseases.
- Where needed, the consent of the former owner (of the donor) and the cryobank.

For convenience, the gene bank may decide to prepare a standard form for requestors to compile, based on the considerations above.

This information will assist the gene bank manage to decide whether or not release of material is justified and beneficial for the national programme of AnGR management.

When germplasm is to be used for animal generation, the gene bank may want to consider requesting that the user redeposit germplasm from the resulting progeny. For germplasm used in DNA studies or live animal generation, it is recommended that any phenotypic or genotypic data be submitted to the gene bank for entry into a publicly accessible database. The submission of such information can take place after the information has been published.

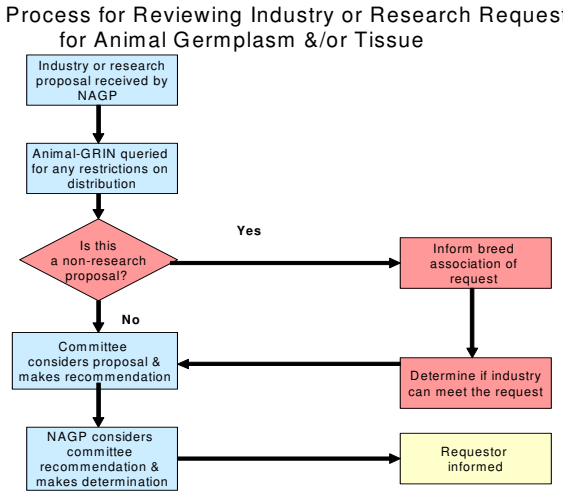
11.3.2. Meeting the Request

Once a request for germplasm or tissue is received and evaluated for its merit, the gene bank must also determine if sufficient quantities of the material exist in the gene bank to fulfil the request without compromising the Core Collection (see Chapter 3). If the requested cryobank material is also available in the commercial sector, the cryobank should not allow use of cryobank material.

National Need: In the case of a national need, the government may decide to withdraw germplasm from the needed categories of the collection. In such a situation the gene bank may want to convene a group of in-country experts and industry related persons to provide recommendations and assist in facilitating the use of the germplasm.

Non-research and Research Requests: The collection can be accessed for non-research and research activities. Non-research requests are usually those made by a segment of the livestock industries for the purposes of resolving a genetic resource issue. Utilization of repository material by research interests (either public or private) is another potential source of requests for this material. For such requests a review process should be established by the gene bank. As an example, Figure 11.1 details the process of reviewing non-research or research requests as used by the United States of America (Blackburn, 2009).

Figure 11.1. Process used by the National Animal Germplams Program (NAGP) in the USA for reviewing industry or research requests for animal germplasm and/or tissue.



11.4. International transfers of germplasm

The primary mission of the national gene bank is to secure the integrity of national AnGR. Therefore, its operation and practices are firmly established under the country's laws. Opportunities may exist to exchange germplasm across national boundaries, in which case the primary regulations involved in such an exchange are animal health concerns. The OIE has established protocols for transferring germplasm from country to country. These protocols have been used by member states of the Sanitary and Phytosanitary measures (SPS) of the World Trade Organisation (WTO) have to establish national SPS measures consistent with internationally 'harmonised' standards, guidelines and recommendations. Beyond health concerns, the exchange of AnGR is mainly through the transfer of private ownership (by private law contracts and customary law). In addition, signatory countries of the CBD need to ensure that any international transfer of AnGR is consistent with the term of the Nagoya Protocol[18].

References

Blackburn, H. D. 2009. Gene bank development for the conservation of livestock genetic resources in the United States of America. *Livestock Science,* 120: 196-203.

Wood, R.J. & Orel, V. 2005. *Genetic Prehistory in Selective Breeding: A Prelude to Mendel.* Oxford University Press, New York, USA. p. 323.

[18] http://www.cbd.int/abs/text/

12. CAPACITY BUILDING AND TRAINING

The development of sustainable conservation programmes is only possible if they are combined with the development of human resources and the building of institutions and the integration of long-term organizational support. Well-trained researchers and decision makers are critical for creating awareness of the problems and for the implementation of programmes to conserve and sustainably use AnGR.

Strategic Priority Area 4 of the *Global Plan of Action for Animal Genetic Resources* (FAO, 2007) calls for development of a strong and diverse skills base to implement the Global Plan at national and international level. The CBD in its Articles 16 to 18 also calls for access to and transfer of technology (Article 16[19]); exchange of information relevant to the conservation, management and use of biological diversity; including information on research, training, surveys, and specialized knowledge (Article 17[20]); and technical and scientific cooperation through, where necessary, appropriate international institutions, with special attention to capacity building (Article 18[21]).

The most important task for the long-term improvement in the knowledge on AnGR will be to make sure that all major aspects of conservation and sustainable use of AnGR are integrated into the regular university curriculum worldwide. Emphasis should be given to global and regional aspects of animal production, considering the importance of interaction effects between different genotypes and environments including among different species within a given environment. Students should be introduced to the agro-ecosystem approach to agriculture and livestock production. A closer collaboration between countries, both developed and less developed, is suggested by extended exchange programmes for students as well as teachers (Malmfors *et al.,* 1994). Vangen and Mukherjee (1994) suggested that an integrated approach to teaching animal breeding and the genetics of conservation should be taken at both undergraduate and graduate levels, with graduate level teaching being the most relevant, as the understanding of the integration will be higher at that level. Training courses should also be organized for national administrators and heads of departments involved in policy decisions, the future decision-makers and facilitators of conservation programmes. The subject matter should give an appreciation of the importance of AnGR, and explain the major steps in their characterization, documentation, conservation and improvement.

The following are among the topics to be taught in higher education, in order to increase the awareness and understanding of the importance of conserving and properly managing AnGR:

Global threats and opportunities of farm animal genetic diversity. This topic should include evolution and the history of domestic species and breeds, the breed and population concepts, animal populations in various parts of the world and present development trends. Livestock production systems in various regions of the world and the prospects and constraints of different animal populations in relation to environmental and socio-economic conditions should also be addressed.

Understanding genetic diversity and factors affecting genetic variation. Education in factors affecting the dynamics of genetic variation in small populations is of great importance. The concept of rate of inbreeding (and hence N_e) and its relation to the dynamics of genetic variance (and other measures) over time is important.

Characterization and documentation of animal populations. In any programme aiming at conservation for future utilization, characterization and documentation of the living populations and the any cryoconserved material is extremely important. It is necessary to know the distribution and trait characteristics in relation to defined environments. It is also important to know how to organize and utilize databanks and descriptors, as well as to monitor population changes and measure genetic relationships between breeds.

[19] http://www.cbd.int/convention/articles/?a=cbd-16

[20] http://www.cbd.int/convention/articles/?a=cbd-17

[21] http://www.cbd.int/convention/articles/?a=cbd-18

Ex situ strategies and methods for conservation of animal genetic resources. Cryopreservation i.e. storage of frozen semen, embryos, oocytes, cell cultures or DNA, including objectives, methods of collection, sample sizes needed and record keeping.

Reproductive biotechnology. Training in reproductive biotechnology will allow countries to undertake programmes for cryoconservation independently. Furthermore it would allow, through technology transfer, to take advantage of the extremely rapid development in the field of advanced biotechnology in developed countries.

Sanitary and legal aspects related to access and exchange of germplasm. Operation of a gene bank requires awareness and knowledge of national and international policies affecting the exchange of AnGR.

References

Malmfors, B., Philipsson, J. & Haile-Mariam, M. 1994. Education and training in the conservation of domestic animal diversity - student needs and field experience. *Proceedings of the 5th World Congress on Genetics Applied to Livestock Production.* Guelph, Canada, 21: 485-492.

Vangen, O. & Mukherjee, T. 1994. Conceptual approach to integrating education in animal breeding and in conservation genetics. *Proceedings of the 5th World Congress on Genetics Applied to Livestock Production,* Guelph, Canada, 21: 477-484.

APPENDIX A. SPECIALIZED PROCEDURES FOR FIELD COLLECTION OF GERMPLASM

(Courtesy of the Canadian Animal Genetic Resources Laboratory for Cryobiology)

A number of separate Standard Operating Procedures (SOP) may come into play for each of the procedures and equipment used to collect semen and embryos. These SOP must be followed appropriately.

Note: If samples will be shipped to the National Gene Bank via courier service, rather than transported by the collection team, contact the nearest courier service company prior to arriving to the collection site and check their latest drop-off times for an overnight delivery to the gene bank. These time limits will dictate the working schedule to collect, initially evaluate, package and deliver the collected germplasm.

When arriving at the site of collection, first introduce yourself to your contact person(s) (owner, breeder, and staff) and clearly discuss the logistical details for safely handling the animals and the collection, handling and analysis of the germplasm to be collected and its delivery to the gene bank.

Be prepared to answer questions and make sure (verbal agreement) that your contact person is comfortable with the procedures and protocols to be used on the selected animals. Make sure the contact person at the collection site duly completes and signs any written agreements involving ownership of the germplasm (e.g. Material Transfer Agreement or Donorship certificate). Also, the technician must sign the agreement prior to starting collection. Make sure a clean and closed-in environment is available to handle and analyze the collected germplasm.

After the logistics are understood and confirmed, wear the necessary personal protective equipment and respect the local biosecurity protocols (if applicable).

Unload and arrange the equipment for collection, handling and analysis of germplasm before proceeding to collection. Follow the appropriate SOP for these steps. Make sure the incubator and necessary media are at the proper temperatures before proceeding.

Use any waiting time (e.g. time required to warm media) to converse with your contact person about the procedures and continue to encourage questions. Make sure your contact person understands you value and care about the donor animals, and that all necessary precautions are being taken to minimize their stress and pain. Explain in detail any special procedures, such as electro-ejaculation or embryo flushing, and how the animal may react. Make sure your contact person is comfortable with the procedures.

When ready to proceed, arrange the collection area. Make sure all necessary precautions are taken to safely constrain the animal and that all persons involved are in a safe working environment.

Proceed with the collection of the germplasm following the appropriate SOP.

When the germplasm is collected, immediately transfer and store the material in the portable incubator and move it to the clean and closed-in area for subsequent procedures (e.g. evaluation, processing and packing or freezing).

Analyze the quality of the collected germplasm under the microscope. Take this time to show your contact person what the sample(s) look like under the microscope. The person may never have seen live embryos or motile sperm cells before. Semen concentration can be evaluated using the haemocytometer.

If a problem is encountered, re-collect the animal.

If the germplasm cannot be transported or shipped immediately to the gene bank, then proceed with the freezing procedures using the appropriate SOP for semen or embryos. If the collected germplasm can be shipped to the gene bank, then proceed with the preparation of the collected germplasm for shipping to the gene bank.

Accordingly, prepare (freeze or pack) the collected germplasm following the appropriate SOP.

For frozen germplasm, use the prepared dry-shipper.

For fresh germplasm, transfer the collected material to 15ml or 50ml tubes, depending on the volume of the collected material.

Place the tube in a reusable and sealable plastic bag containing shredded absorbent paper and seal the plastic bag.

Place frozen ice packs on the bottom of the shipping container and cover with several layers of absorbent paper (1cm thick) to avoid direct contact of the germplasm with the ice pack.

Place the sealed plastic bag containing the germplasm on top and cover with several layers of absorbent papers. Secure the shipping container with packaging tape. The shipping container must be spill-proof to be accepted by the courier service company.

Place a shipping label (addressed to the National Gene Bank) on the shipping container and contact the courier service company for pick-up and delivery or deliver it yourself.

Upon arrival of the collected germplasm at the genebank, a sub-sample is analyzed to determine the viability of the material after the cryopreservation process, following the appropriate SOP. A brief report, including viability, quality and the number of straws processed, is prepared and sent back to the contact person at the collection site.

APPENDIX B. EQUIPMENT NEEDED FOR SEMEN COLLECTION AND FREEZING

1. Major equipment and facilities

- Animal handling facilities
- Adequate dedicated workspace for semen processing (clean, dry, climate controlled)
- Warming cabinet (electric)
- Microscope (10X eyepiece with 10 and 43X objectives)
- Digital balance (6 kg ± 1 g)
- Warm water bath (electric)
- Sperm counting equipment (haemocytometer or spectrophotometer)
- Straw filler (preferably with attached label printer)
- Semen freezing unit
- Semen liquid nitrogen (LN2) storage tanks (pre-tested)
- Source of LN2 (very important to have a reliable source year around)

2. Small equipment and consumables

- Coveralls and boots
- Disposable gloves and boot covers
- Helmet (head protection), especially for semen collection form stallions
- Artificial vaginas, cones and collection tubes
- Lubricant
- Glass-Lined thermos bottles
- Thermometers (centigrade)
- Semen diluents, extenders and cryoprotectants
- Plastic semen straws
- Assorted glassware and plasticware
- Artificial light

APPENDIX C. PROCEDURES FOR CRYOPRESERVATION AND THAWING OF SEMEN FROM COMMON ANIMAL LIVESTOCK SPECIES

The following procedures require technical expertise. Before widespread implementation in a national conservation programme, the gene banking team should test the procedures on a small group of animals (preferably of the same breed). The test should include both semen collection and freezing and thawing and insemination to obtain a successful pregnancy.

Cryopreservation of Bull Semen

For a review of semen collection, processing and handling for bulls see the classic laboratory manual by Herman *et al.* (1994).

Freezing

1. The collected sperm should contain approximately 5 to 15 x 10^9 sperm per ejaculate. Avoid temperature changes in semen during transfer to the processing laboratory. In the laboratory, use a water bath to maintain the temperature at approximately 35°C.
2. Visually inspect the semen to ensure proper colour (usually milky white) and freedom from abnormalities (e.g. blood, pus). Filter the semen if contaminants such as hair or dung are visible.
3. Microscopically evaluate semen to evaluate motility (>60%) and proportions of live sperm (>70%) and abnormal (<30%) sperm (See Appendix D).
4. Evaluate sperm concentration (haemocytometer or spectrophotometer) and determine final volume V for a diluted concentration of ≥100 x 10^6 sperm/ml.
5. Dilute the semen to the proper volume using One-Step or Two-Step procedure:
   a) One Step procedure
      - Add the entire sample to the corresponding volume of One-Step Diluent (20% egg yolk, 7% glycerol plus antibiotics to yield a final concentration of 100 µg of tylosin, 500 µg of gentamicin, 300 µg of lincomycin, and 600 µg of spectinomycin in each ml of total volume) to obtain the desired final sperm concentration.
      - Cool to +5°C within 1 hour and maintain for at least 2 hours.
   b) Two Step procedure
      - Add half of the final volume with Diluent A (milk +10% egg yolk + antibiotics + 3% glycerol). Addition must be done progressively over 15 min at 35°C.
      - Cool to +5°C within 1 hour.
      - Add Diluent B up to final volume (Diluent B consists of Diluent A +11% glycerol).
      - Keep at +5°C for 2 hours.
   c) For the above methods, protein-free commercially available products without milk or egg yolk are available, which can reduce sanitation risks.
6. Fill pre-printed straws of 0.25 ml with semen (25 to 30 million sperm/straw).
7. Transfer straws to LN2 vapour at -70°C/-100°C for 9 minutes.
8. Transfer straws to LN2 tank and store.

Thawing

1. Before inseminating animals from a given batch of semen, thaw one straw to check for quality.
2. Thaw straw directly in a water bath at +37°C for 30 seconds.

3. Inseminate cows and heifers transcervically ~12 hours after onset of standing estrus.

Crypopreservation of Buffalo Semen

Freezing

1. Collect sperm (5 to 10 x 10^9 sperm per ejaculate) at 35°C and transfer to the laboratory.
2. Avoid temperature changes in semen after collection by placing sample in water bath (35°C).
3. Evaluate sperm visually and microscopically.
4. Measure concentration and determine final volume V for a concentration of 100 x 10^6 sperm/ml.
5. Add half of the final volume with Diluent A (milk + 10% egg yolk + antibiotics + 3% glycerol). Addition must be done progressively over 15 minutes at 35°C.
6. Cool to +4°C within 1.5 hours.
7. Add Diluent B up to final volume (Diluent B consists of Diluent A + 11% glycerol). The final concentration of glycerol is thus 7%.
8. Keep at +4°C for 4 hours.
9. Meanwhile, fill pre-printed straws of 0.50 ml with semen (about 50-60 million sperm/straw).
10. Cool from +4°C to -140°C in 5 minutes in LN2 vapour, then plunge in LN2.
11. Transfer straws to LN2 storage.

Thawing

1. Thaw a sample from each batch to check for quality prior to use for AI.
2. Thaw straw directly in a water bath at +35°C for 30 seconds.
3. Inseminate females transcervically 12 hours after onset of oestrus.

Cryoconservation of Ram Semen

Freezing

1. The collected semen should contain about 4 x 10^9 sperm per ejaculate and should be maintained at 37°C.
2. Evaluate semen visually and microscopically. Semen should be white and quite viscous.
3. Select only those ejaculates with mass motility >10% and <30% abnormal sperm.
4. Evaluate sperm concentration and determine final volume V for a concentration of 400 x 10^6 sperm/ml.
5. Dilute the semen to the proper volume using One-Step or Two-Step procedure. For a discussion of semen diluents and extenders for ram semen, see Paulenz *et al.,* (2002).
   a) One Step procedure
      - Add the entire sample to the corresponding volume of One-Step Diluent (300mM Tris, 28 mM glucose, 95 mM citric acid, 2% (v:v) glycerol, 15% egg yolk, 1 mg/ml of streptomycin sulfate and 0.06 mg/ml of benzylpenicillin) to obtain the desired final sperm concentration.
      - Cool to 4°C within 1 hour and maintain for at least 1.5 hours.
   b) Two Step procedure
      - Add to the semen Diluent A at 30°C to obtain 60% of the final volume. (Diluent A consists of 25.75 g of lactose in 250 ml bi-distilled water +20% egg yolk.)
      - Cool progressively to +4°C over 2 hours (0.2°C/min).

- Prepare Diluent B: Reconstitute milk from a non-fat powder source (4 g into 100ml bidistilled water) and adjust pH to 6.6 with a Tris solution (20 g of tri-sodium-citrate-5.5H2O into 70 ml H2O); then mix 9 volumes of the resulting solution with 1 volume of glycerol.
- Add Diluent B up to final volume (Diluent B consists of Diluent A +11% glycerol).
- Add Diluent B in 3 equals parts, over 30 minutes, at 4°C up to the final volume.
- Keep the semen for 90 minutes at +4°C.

6. Fill semen into 0.25 ml plastic straws.
7. Place straws horizontally in LN2 vapour at -75°C for 8 minutes.
8. Transfer directly into LN2 at -196°C and store.

Thawing

1. Thaw straws in a water bath at 37°C for 30 seconds.
2. Assess semen viability: mix one volume of sperm to 4 volumes of a sodium citrate solution (20 g of Tri-sodiumcitrate-2H_20 in 70ml bi-distilled water) at 38°C and estimate the proportion of motile sperm 5 minutes and 2 hours after: only sperm with more than 30% of living spermatozoa (spz) at 2 hours can subsequently be used for insemination.
3. Proceed to surgical or non-surgical insemination of pre-synchronized recipient.

Cryoconservation of Buck Semen

Freezing

1. Collected semen should contain about 4 x 10^9 sperm per ejaculate in season. Semen should be kept at 32°C for transfer to the laboratory and processing.
2. Evaluate semen visually for any abnormalities.
3. Wash sperm with a Krebs Ringer Phosphate Glucose Solution (0.9%NaCl; 1.15% KCl; 1.22% $CaCl_2$; 2.11% KH_2PO_4; 3.82% $MgSO_4$-7H_2O; 5.24% glucose) by mixing one volume sperm with 9 volumes of the washing solution at 28-32°C, followed by a centrifugation at 500 g for 15 minutes at 20°C.
4. Discard the supernatant, and evaluate semen (wave motion, concentration). Select only those ejaculates with a mass motility ≥60%.
5. Calculate final volume (V). Repeat centrifugation under same conditions at 20°C.
6. Dilute the semen to the proper volume using One-Step or Two-Step procedure:
   a) One Step procedure
      - Add the entire sample to the corresponding volume of One-Step Diluent (300mM Tris, 28mM glucose, 95mM citric acid, 2% (v:v) glycerol, 2.5% egg yolk, 1 mg/ml of streptomycin sulfate and 0.06 mg/ml of benzylpenicillin) to obtain the desired final sperm concentration.
      - Cool to 4°C within 1 hour and maintain for at least 1.5 hours.
   b) Two Step procedure
      - Prepare Diluent A: 80 ml of a sodium citrate solution (194 mg glucose +3.52g sodium citrate +1.05g streptomycin +50 000 IU penicillin in 100 ml distilled water) supplemented with 20 ml egg-yolk.
      - Add V/2 of Diluent A to the pelleted sperm at 20°C.
      - Cool to +4°C within 30 minutes (at 0.5°C/min).
      - Add V/2 Diluent B (Diluent A + 14% v/v glycerol) in three successive steps with 10 min intervals. Diluent should also be at +4°C.

7. Fill 0.25 ml plastic straws with semen.
8. Freeze straws in LN2 vapour for 5 minutes.
9. Plunge directly into LN2 and store.

Thawing

1. Thaw straws in a water bath at 37°C for 30 seconds.
2. Assess post-thaw motility.
3. Proceed to insemination of does.

Cryoconservation of Stallion semen

Freezing

1. Collect sperm (~8 x 10^9 sperm per ejaculate) and filter on gauze.
2. Dilute semen to an approximate concentration 50 x 10^6 sperm per ml of in 37°C holding medium. Various suitable media can be used. One example SMED (100 ml of nanopure water, NaCl at 37 mM, KCl at 10 mM, KH_2PO_4 at 0.07 mM, $NaHCO_3$ at 35.7 mm, $MgSO_4$ at 2.4 mM, HEPES at 10mM, $CaCl_2$ at 1.7mM, fructose at 84.3 mM and glucose at 5.5 mM supplemented with 0.3 g of bovine serum albumen at a pH of 7.2).
3. Centrifuge the extended semen (800 x g for 9 minutes or 400 x g for 13 minutes)
4. Remove the supernatant by aspiration.
5. Suspend the resulting pellets in a small amount of SMED and determine the sperm concentration.
6. Redilute the sample to 400 x 10^6 sperm per ml in skim milk-egg yolk extender (SMEY) (154.8mM glucose, 4.2 mM lactose, 0.5mM raffinose, 0.85mM sodium citrate dihydrate, 1.25mM potassium citrate, 29.8mM HEPES, 51.5 mg/ml of skim milk powder, 1 mg/ml of ticarcillin with 2% egg yolk)
7. Cooled to 15°C in a shipping container, which will allow it to be held up to 24 hours.
8. After holding, further cool the sample to 5°C over 2 hours.
9. Dilute with SMED-glycerol, so that the final dilution is 4% glycerol (up to a 1:1 dilution [v:v]).
10. Load the samples into printed straws.
11. Freeze in LN2 vapour (4.5 cm above the liquid for 10 minutes)
12. Plunge into LN2 for storage.

Thawing

1. Thaw eight 0.5 ml straws (400 x 10^6 sperm) together in a water bath at +37°C for 30 seconds.
2. Sperm should be deposited usually daily into the uterine body during the estrous period.

Cryoconservation of Boar Semen

Freezing

1. When collecting boar semen, it's critical to discard the first emission of sperm while keeping only the second (about 200 ml, rich in sperm with 40 x 10^9 total).
2. Filter the semen through gauze to eliminate the bulbo-urethral secretions.
3. Semen should be kept at 37°C until processing, which should be done as quickly as possible. Ideally, semen will be extended within 15 minutes.
4. Two approaches can be applied, depending if collection is in the field or laboratory:
   a) In the field

- If semen is collected in the field, the ejaculate should be quickly diluted in 37°C Beltsville Thawing Solution (BTS) (205 mM of glucose, 20.4 mM of sodium citrate, 14.9 mM of NaHCO3, 3.4 mM of EDTA and 10 mM of potassium chloride), placed in the shipping container and cooled to 15°C.
- Upon arrival at the laboratory, centrifuge the samples at 800 x g for 10 minutes.
- Combine the pellets and determine the sperm concentration.
- Dilute samples using BF5 Cooling Extender (CE) (52mM of TES, 16.5mM of Tris [hydroxymethyl] aminomethane, 178 mM of glucose with 20% egg yolk (at 325 mOsm) to 750 x 106 sperm per ml.
- Cool semen to 5ºC over 2.5 hours.
- Diluted semen with BF5 freezing extender (91.5% of CE, 6% glycerol, 2.5% Equex Paste [v:v]) to 500 x 106 sperm per ml.

b) At a semen collection and processing centre

- Dilute one volume of sperm with one volume of Diluent A (anhydrous dextrose, 37g; tri-sodium-citrate-2H2O, 6 g; NaHCO3, 1.25 g; EDTA diNa, 1.25 g; KCl, 0.75 g in 1 litre of bi-distilled water).
- Cool to 15°C within 2 hours.
- Centrifuge at 800 x g for 20 minutes at 15°C.
- Remove supernatant, which is diluted seminal plasma.
- Resuspend the pellet of sperm with about 10 volumes of Diluent B (fructose, 8.5 g; of NaHCO3, 0.15 g; of cysteine, 0.015 g; bi-distilled water, 116 ml; egg yolk, 34 ml; equex STM [Nova Chemicals], 1.69 g) to obtain a concentration of 3 x 109 sperm per ml.
- Cool the suspension to 5°C over 2 hours.
- Add one volume of the diluted sperm solution to one volume of Diluent C (Diluent C consists of Diluent B +6% glycerol); Diluent C must be added in 3 steps to give a final concentration of 3% glycerol and 1.5 x 109 sperm per ml.
- Keep at 5°C for about 90 minutes.

5. Load the semen into 0.5 ml. straws.
6. Place straws horizontally at 5 cm above the level of boiling LN2 for 15 minutes (this will ensure a freezing rate of about 20°C/minute down to -145°C.
7. Plunge into LN2 and store.

Thawing

1. Thaw straws in a 38°C water bath for 20 seconds.
2. Mix the content of 7 straws with 95 ml of Diluent A at 38°C to obtain one dose for one AI.
3. Inseminate the sow within one hour after this dilution (5.3 x 10^9 sperm per AI).
4. See also Pursel *et al.,* (1975) and Almlid &Johnson (1987) for more information.

Cryoconservation of Rabbit Semen

Freezing

1. Collect semen.
2. Prepare Diluent A. For 100 ml bi-distilled water dissolve: 3.028 g of Tris (trishydroxy-methylaminomethane) (Tris); 1.25 g of glucose; 1.67 g of citric acid-H_20; 5 ml of DMSO (dimethyl-sulfoxide); add 1 volume egg yolk for 4 volumes solution (Vicente & Viudes-de-Castro, 1996).

3. Add 4 volumes Diluent A to one volume sperm
4. Progressively cool the diluted semen to +5°Cover 1 to 3 hours.
5. Prepare Diluent B. For 100 ml of bi-distilled water dissolve: 8.25 g of lactose; 1.3 ml of glycerol; add 20% egg yolk (1 volume egg yolk for 4 volumes of solution).
6. Add one volume of Diluent B pre-cooled at +5°C to one volume of diluted semen
7. Fill 0.5 ml straws with semen.
8. Keep 10 minutes at +5°C.
9. Freeze straws horizontally in LN2 vapour for 3 minutes at -120°C.
10. Plunge directly into LN2 and store.

Thawing

1. Thaw straws in a water bath at +37°C for 1 minute.
2. Intravaginal insemination of does followed by an intra muscular injection of 0.2 ml Gonadotropin Releasing Hormone (GnRH).

Cryopreservation of Rooster Semen

Two methods for cryopreservation of poultry semen are presented. The primary difference is the media used.

Method I

With this method the semen can be used directly for insemination after thawing. No need to first wash the semen free of the cryoprotectant.

Freezing

1. Prepare media.

Base Medium: Lake's diluent described in Lake (1968) as "Solution 1"

	M.W.	g/l	mmol/l
Sodium-L-glutamate.H_2O	187.13	19.2	102.6
Magnesium acetate4H_2O	214.46	0.7	3.3
Fructose	180.16	8	44.4
Potassium acetate	98.2	5	50.9
Polyvinylpyrrolidone	40 000	3	0.08

- Final pH = 6.9
- This medium is prepared with and without cryoprotectant DMA (dimethylacetamide)
  - Lake
  - Lake-DMA
- DMA concentration is 1.8 mol/l = 157 g/l = 16.7 vol %
- Take 15.7 g (or 16.7 ml) of DMA and add Lake's diluent to a total volume of 100 ml.

2. Store media in closed vessels to prevent evaporation and place in a temperature-controlled cool box at 5°C.
3. Collect semen and transport to the laboratory for further processing and freezing. All further handling is performed at 5°C (cold room or open top cooler cabinet).
4. Determine the concentration of the non-diluted semen (spectrophotometer or haemocytometer).
5. Diluted semen with Lake's diluent to the desired concentration (e.g., 1.8 x 10^9 sperm/ml).
6. Add half a volume of Lake-DMA to one volume of semen and fill into 0.25 ml straws.

7. Freezing may be performed in a programmable freezer with a constant rate of 50°C/minute) (maximum rate of most freezers) or in static LN2 vapour, 1-2 cm above the LN2 level.

Thawing

1. Remove straws are taken from LN2 and place in a 5°C water bath.
2. Move the straws vigorously through the water for 30 seconds. Do not thaw bundles of straws as this will slow down the thawing rate. Despite the low temperature of the water bath, the thawing rate is still high enough (average thawing rate between ± −190 and + 5 °C is 500-600 °C/minute).

Method II

With this method the cryoprotectant must be washed from the thawed semen prior to insemination.

Freezing

1. Collect sperm (1.5 x 10^9 sperm per ejaculate).
2. Mix three volumes of sperm (an ejaculate is about 300 µl) with four volumes of Diluent A (0.7 g of magnesium acetate (tetra-hydrated) + 19.2 g of sodium glutamate + 5.0 g of sodium acetate + 8.0 g of fructose + 3.0 g P.V.P (MW 10 000 to 15 000) in one litre of bi-distilled water.
3. Cool diluted semen immediately over 20-30 minutes to +5°C (0.5°C/min).
4. At +5°C, add one volume of diluted sperm to one volume of Diluent B (Diluent A +11% glycerol). This gives a final concentration of 300 x 10^6 sperm/ml.
5. Equilibrate over 30 minutes at +5°C.
6. Fill 0.25 ml straws with semen.
7. Freeze at a rate of 7°C/minute from +5°C to -35°C; and at a rate of 8°C/min from -35°C to -140°C.
8. Plunge into LN2 and store.

Thawing

1. Prepare Diluent C: 0.8 g magnesium acetate (tetrahydrated) +1.28 g of potassium citrate +19.2 g of sodium glutamate +6.0 g of fructose +5.1 g of sodium acetate in one litre of bi-distilled water.
2. Prepare Diluent D: 0.8 g of magnesium acetate (tetrahydrated) +1.28 g of potassium citrate +15.2 g of sodium glutamate +6.0 g of glucose +30.5 g of B.E.S (N,N-bis-2 hydroxyethyl-2-amino-ethanesulfonic acid) +58 ml of NaOH (1M/l) in one litre of bi-distilled water.
3. Thaw straws in a water bath at +5°C for 3 minutes. Open and transfer semen in a glass beaker. Mix one volume of sperm with 20 volumes of Diluent D, still at 5°C.
4. Remove glycerol by centrifugation at 700 g at +5°C for 15 minutes
5. Discard the supernatant and add one volume of sperm pellet to one volume Diluent D at +5°C, and proceed to insemination of the hens.

Cryoconservation of Turkey and Duck Semen For the present it is recommended that turkey and duck semen samples are treated as rooster semen, however, additional improvements are forthcoming (Woelders, 2009). For insemination of turkeys, 3 straws per insemination are recommended.

APPENDIX D. GENERAL GUIDELINES FOR BASIC SEMEN ANALYSIS

Cryopreservation of semen can be a valuable step in ensuring the long-term survival of a given AnGR. However, these efforts will be futile if and a waste of resources if the sperm is not fertile. Therefore, collected semen should be evaluated for quality and viability prior to processing and freezing

Semen should be evaluated as soon after collection as possible. Exposure to temperature changes, light and contaminants are generally detrimental to semen quality. Specific equipment, such as computer-aided semen analysis (CASA) systems can be used for automated evaluation of motility parameters, but subjective evaluation under a microscope is also done and technicians should be trained in these techniques.

Three basic characteristics should be evaluated when evaluating semen and estimating sperm viability:

1. Sperm concentration
2. Motility
3. Morphology

Sperm concentration

Concentration is most accurately estimated with specialized equipment such as a spectrophotometer. Counting can also be done manually, under the microscope, using a haemocytometer. A haemocytometer is a thick glass slide with two vessels serving as counting chambers. Each chamber is marked with a grid pattern etched into the glass, creating a background of squares. Diluted semen (usually 1:100) is pipetted into the chambers and the haemocytometer is viewed under a microscope. Because counting is facilitated and more accurate when the sperm are immobile, sperm are usually killed by including a small quantity of formaldehyde in the diluent. By counting the number of sperm with a sample of squares in the grid and considering the size of the squares of the grid and dilution rate, the concentration and number of sperm in the original sample can be estimated. This information can then be used to determine the proper quantities of semen extender needed to obtain the desired concentration of sperm in the semen to be packed and cryopreserved. Sperm concentration can also be used as an indicator of the health of the semen donor (low concentration may indicate a health problem).

Motility

The movement of the sperm should be checked; first, because observation of movement ensures that the sperm are alive, and second, because motility is correlated with fertility. Two types of motility are usually evaluated, 1) gross motility and 2) individual motility.

1. Gross motility
   - Place drop of diluted semen on a pre-warmed slide (37°C) and examine sperm at 10X under a standard or phase contrast microscope.
   - Look for general movement of the sperm with rapidly moving waves and individual swirls of sperm within the waves.
2. Individual motility
   - Place on a pre-warmed slide a drop of semen diluted (1:10) in saline solution, citrate or extender. When CASA equipment is used, chambers of a special design are needed (i.e. Makler chamber).
   - Position a cover slip over the mixture and examine under ≥40X magnification.
   - Estimate the proportion of individual sperm that are moving progressively forward (i.e. “progressive forward motility) – this can be done by randomly picking 10 or more sperm in different areas of the slide, counting those with forward motility and dividing by the number observed.

- Although motility and its correlation with fertility can vary by species, the following can be used as a general guideline:
    - >70% = very good
    - 50 to 60% = good
    - 40 to 50% = satisfactory
    - 30 to 40% = acceptable, but undesirable
    - <30% = unsatisfactory

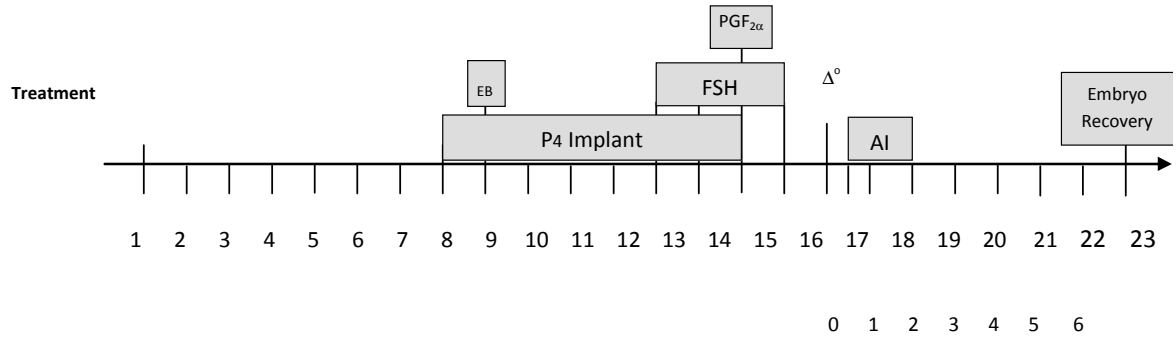
Morphology

Abnormally shaped or damaged sperm are less likely to be capable of fertilization than are normal sperm (Berndtson *et al.,*1981). Mixing the semen with a stain (e.g. eosin-nigrosin) highlights the sperm so that abnormalities can be readily identified under a microscope. Two kinds of abnormalities can be defined: *primary* abnormalities, which are assumed to have occurred in the testes and *secondary* abnormalities, which arise in the epididymis or ejaculate. The proportion of normal sperm should be >70%.

1. Place a drop or stripe of stain on a warmed microscope slide.
2. Add a small amount of semen.
3. Mix the semen and stain with another slide and then use the narrow edge of the second slide to smear the mixture across the initial slide.
4. Cover with cover slip and examine under 1000X magnification (oil immersion).
5. Examine the sperm for abnormalities, including the following:
    - Abnormally shaped (tapered or pear-shaped) or sized (too large or small) heads
    - Missing or stump tails
    - Coiled or bent tails
    - Detached or creased (folded-over) acrosome
    - Clumping of multiple sperm
    - Plasma droplets on tails
6. Count at least 100 sperm and calculate the proportion (%) of abnormalities.
7. Discard semen if proportion of abnormalities is too high (>30%).

APPENDIX E. COMMONLY USED SUPEROVULATION SCHEMES FOR DONOR CATTLE EMBRYO RECOVERY

Scheme I



$PGF_{2\alpha}$ Prostaglandin F_2 alpha.

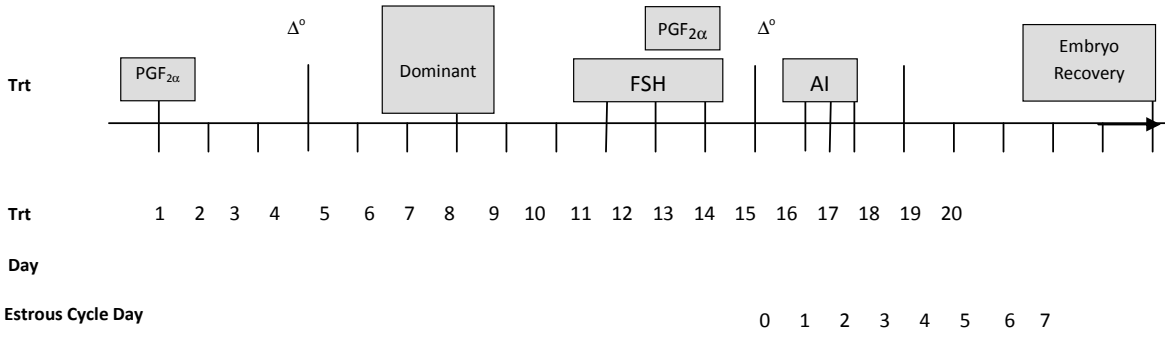
EB = 2 to 5 mg of estradiol benzoate injected. This agent may be illegal to use in some countries.

Δ^o = Standing estrus.

P4 Implant = Intravaginal progesterone implant (commercially available in most countries).

FSH = Follicle Stimulating Hormone

Scheme II



APPENDIX F. EQUIPMENT AND SUPPLIES NEEDED FOR NONSURGICAL EMBRYO COLLECTION AND TRANSFER IN CATTLE.

1. Equipment:

- Animal holding chute
- Temperature controlled water bath
- LN2 tank
- Stereomicroscope with a heated stage
- Cassou gun and sheaths

2. Renewable Supplies:

- Boots (washable)
- Coveralls (washable or disposable)
- Plastic gloves (disposable)
- Plastic foot covers
- Paper towels
- Disinfectant liquid
- Liquid soap
- Petri dishes
- Small sterile plastic embryo dishes
- Scissors
- Lidocane
- Donor semen (2 to 4 units)
- Plastic syringes 10 or 12 ml and needles for tailblock procedures
- Flushing medium (Delbecco's phosphate-buffered saline)
- Holding medium (e.g., TCM-199 a commercially available medium)
- Fetal calf serum (commercially available)
- Plastic straws for the embryos (e.g., 0.25 ml)
- Straw labeling equipment (preferably with label printer recommended)
- LN2
- Record book (Very important!)

APPENDIX G. TECHNICAL PROCEDURES FOR CRYOPRESERVATION AND THAWING OF FARM ANIMAL EMBRYOS

It is anticipated that the team responsible for the cryopreservation and/or thawing of embryos will have demonstrated their technical expertise before implementing an embryo recovery and banking program. Freezing cattle, goat and sheep embryos has been successful and is presently common for in-field use. Freezing early-stage horse embryos has been reported but freezing later-stage equine embryos has still has not been widely mastered. Freezing swine embryos is more difficult, although there have been some recent advances in this area. For these reasons, only procedures for ruminant and horse embryos are outlined here.

Cryopreservation of Bovine Embryos

Freezing

1. Embryos from a superovulated donor female are collected nonsurgically at day 7 of the estrous cycle, evaluated for morphology development and assigned an embryo quality grade. The embryos should be at the compact morula and blastocyst stages, if everything with the procedure is progressing correctly.
2. Maintain the embryos in a clean environment free from possible contaminants and maintain the environment at around room temperature (20 to 30°C). Freezing should be done as soon as possible after collection (i.e. within 4 to 6 hours).
3. While grading quality, ensure that the zona pellucida is intact on all embryos (under 50X light microscope) and that embryos are free from adherent material.
4. Wash the embryos from one donor (nore more than 10 embryos) in 5 consecutive baths of phosphate-buffered saline (PBS) solution containing broad spectrum antibiotic and 0.4% bovine serum albumin. Use different glass and plastic ware for each donor and new micropipettes for each subsequent wash.
5. Treat the embryos to two washes with trypsin (60 to 90 seconds in total) to remove or deactivate any viruses. Trypsin wash is sterile porcine-origin trypsin (1:250) in Hanks' balanced salt solution at a concentration of 0.25%.
6. Wash the embryos an additional 5 times in PBS - antibiotic solution with 2% bovine serum albumin.
7. Equilibrate the embryos at room temperature for 10 minutes in PBS with 10% fetal calf serum (FCS) and 10% glycerol.
8. Place the embryo between two or four air bubbles in a 0.25 ml sterile, pre-labelled plastic straw. Most often one embryo is cyropreserved per straw.
9. Place straws horizontally in a freezing unit and cool from room temperature to -7°C at a rate of 5°C/min.
10. Induce seeding at -7°C by contact by a LN2 cooled tweezers at the extreme end of the straw and freeze the embryo to -35°C at a rate of 0.5°C/min.
11. Plunge directly into LN2 and then store straws in LN2 at -196°C.

Thawing

1. Select the appropriate straw from the LN2 storage tank. *Important!* Do not bring the straws up above the frost line of the LN2 tank (neck of the tank) until the correct straw is identified for embryo transfer.
2. Thaw the straw rapidly in a water bath at 20°C for 30 seconds or 39°C for 8 to 25 seconds, depending on the initial embryo freezing rate. Then cut the ends of the straw and remove the embryo. Rehydrate the embryo in 1 Molar (M) sucrose solution for 10 minutes then reducing the sucrose concentration in a stepwise procedure.

3. Prepare the Cassou gun and clean the perineal region of the recipient. Transfer the contents from one straw (one embryo) to the uterine horn corresponding to the CL of a day-7 recipient female.

Cryopreservation of Goat and Sheep Embryos

Freezing

1. Follow steps 1 to 6 of the bovine protocol.
2. Equilibrate embryos collected from one donor female at room temperature for 10 minutes in PBS with 10% FCS and 10% cryoprotective agent. Ethylene glycol can be used as a cryoprotectant for sheep whereas glycerol is often used for goats.
3. Place one or two embryos between 2 or 4 air bubbles in a 0.25 ml sterile pre-labelled plastic straw.
4. Place the plastic straws horizontally in the freezing unit and cool from room temperature to -7°C at the rate of 5°C/minute.
5. Induce seeding at -7°C and freeze embryos to -30°C at a rate of 0.3°C/min.
6. Plunge straws directly into LN2. Store the straws in LN2 at -196°C.

Thawing

1. Select the appropriate straw from the LN2 storage tank. *Important!* Do not bring the straws up above the frost line of the LN2 tank (neck of the tank) until the correct straw is identified for embryo transfer. Thaw one straw at a time and transfer the embryo(s) before thawing the next straw.
2. Thaw the straw rapidly in a water bath at 20°C for 30 seconds. Then cut the ends of the straw and remove the embryo. Rehydrate the embryo in 0.5M (molar) sucrose solution for 10 minutes then reducing the sucrose concentrations in a stepwise procedure.
3. Proceed to transfer into a prepared female.

Cryopreservation of Horse Embryos

Freezing

1. The following procedure is the slow-freezing approach of Czlonkowska et al. (1985). Commercial vitrification kits are also an option.
2. Collect embryos approximately 6 to 7 days following insemination and transport to the processing laboratory and wash in PBS solution.
3. Equilibrate embryos collected from one donor female at room temperature by washing 4 separate times (for 10 minutes each wash) in solutions of 2.5, 5, 7.5 and 10% glycerol in PBS.
4. Load each equilibrated embryo into a 0.25 ml straw in a drop of 10% glycerol solution between two air bubbles.
5. Seal the straw and place vertically in a freezing rack.
6. Cool straw from room temperature to -6°C at rate of 1°C /min.
7. Hold straw at -6°C for 5 minutes and then seed with forceps cooled in LN2.
8. Freeze straw by reducing temperature to -33°C at rate of 0.3°C /min.
9. Hold straws at -33°C before plunging in LN2.
10. Store straws in LN2.

Thawing

1. Remove straw(s) from the tank and expose them to air for 10 s.
2. Submerge straws for 1 min in 35°C water bath.

3. Expel embryo into a PBS solution containing 10% glycerol and 0.25M sucrose.
4. Wash out the cryoprotectant was then washed out by incubating the embryos for 10 min each in solutions of 0.25M sucrose in PBS with progressively decreasing concentrations of glycerol (7.5, 5, 2.5 to 0%).
5. Proceed to embryo transfer.

APPENDIX H. BOVINE OOCYTE COLLECTION PROCEDURES

Section H1. Collection from dissected ovaries (Technique 1 - Slashing)

1. Materials

- Oocyte collection medium (OCM) – See Table H1
- L-Glutamine
- BSS + Heparin
- Pen/Strep
- 1x saline solution (0.9%) – See Tables H3 and H4
- Petri dishes
- Bench top paper
- 400 ml beaker
- Scalpel handle
- Scalpel blades (sizes #11 and #20)
- Haemostat
- Mouth pipette (optional)

2. Preparations

a. OCM + Supplements (OCM+) – See Table H2

i. Prepare OCM+ by adding the following to one litre of OCM:
- BSS + Heparin (Stock) 20 ml
- Pen/Strep (e.g. Gibco 15140-122) 10 ml
- L-Glutamine (e.g. Gibco 25030-081) 10 ml

ii. Place OCM+ at room temperature at least two hours before arrival of ovaries.

b. Set Up for Collection (1 station per person)

iii. Cover bench top with paper.

iv. Place the following on the bench top:
- 400 ml Beaker
- Scalpel handles
- Scalpel blades (sizes #11 and #20)
- Haemostats
- 1x saline brought to room temperature to wash ovaries

3. Procedures

a. Clean the ovaries with 1X saline solution

b. Slashing

i. Add 150 ml OCM+ to each beaker.

ii. Attach a haemostat to the base of the ovary. Cut the excess tissue away from the ovarian stalk by using #21 scalpel blade and blot off blood with absorbent tissue.

iii. Slash follicles in the size range of 2-8 mm. Hold the ovary above the beaker and make several small incisions to each follicle using the #11 scalpel blade. Both follicular fluid and blood in the collection medium could result in clotting of the medium, thereby rendering it impossible to retrieve oocytes. To prevent clotting of the medium, avoid slashing large follicles (>10 mm) and corpora lutea. Once all of the follicles on the ovary are slashed in one direction, go back over it and slash each in the opposite direction, making an

X through each follicle. This effectively opens the follicles and allows the oocytes to be washed out.

iv. Submerge the ovary into OCM and swirl it several times. Repeat this process until 10 ovaries have been processed/person.

c. Searching

i. Once a group of ovaries have been processed, fill the beaker with OCM+ and incubate at room temperature for five minutes to allow oocytes to settle.

ii. Bathe the outside of the beaker with Ethanol and transfer the beaker to the hood and allow oocytes to settle again for a few minutes.

iii. Using aseptic technique, slowly aspirate OCM from the top of the beaker down to 50 ml. Be careful to not disturb the oocytes on the bottom of the beaker. Stop immediately if this should occur, and allow the oocytes to settle again. Fill the beaker again with OCM+ and let settle for another 5 minutes, and slowly aspirate down to 50 ml.

iv. Transfer remaining media, with oocytes, to two grid plates. Wash the beaker with about 20 ml of OCM and add to the grid plates.

v. Collect cumulus oocyte complexes (COC) as fast as possible using a mouth pipette. Place retrieved COC into the first Petri dish containing OCM+ for further washing.

vi. Transfer COC from first dish to the next leaving all debris behind (repeat twice to assure that oocytes are clean of debris).

Video demonstrations of the above techniques can be found at http://www.animal.ufl.edu/hansen/ivf/Videos.htm, courtesy of Dr. P. Hansen, University of Florida.

Section H2. Oocyte Collection (Technique II - Aspiration)

1. Materials

- Holding Medium (HM) – See Table H5
- 1x saline solution (0.9%)
- Petri dishes
- Bench top paper
- 50 ml conical tube
- 10 ml plastic syringe
- 20-22 gauge needle
- Mouth pipette (optional)

2. Preparations

a. Set up for collection (1 station per person)

i. Cover bench top with paper.

ii. Add the following to the bench top:

- 50 ml conical tube
- tube holder
- 10 ml air syringe
- 20-22 gauge needle

b. 1x saline brought to room temperature to wash ovaries

3. Procedures

a. Clean the ovaries with 1x saline solution

b. Aspiration

i. Use the syringe to aspirate every follicle in the size range of 2-8 mm.

ii. Deposit the follicular fluid slowly in the 50 ml conical tube.

iii. After aspirating all the ovaries allow the oocytes to settle.

iv. Using Pasteur pipette, slowly aspirate the oocytes from the bottom of the 50 ml tube. Be careful not disturb the oocytes.

v. Place the oocytes in a grid dish containing enough holding medium (HM) to cover the dish.

vi. Wash the oocytes with holding medium three times.

vii. Collect cumulus oocyte complexes (COC) as fast as possible using a mouth pipette. Place retrieved COC into the first Petri dish containing OCM+ for further washing.

viii. Transfer COC from first dish to the next leaving all debris behind (repeat twice to assure that oocytes are clean of debris).

4. Media Preparation

Table H1. Oocyte Collection Medium (OCM) (without Supplements)

Ingredient	Quantity/L	Location
M 199 w/ Hank's Salts (e.g. Sigma M-0393)	1 bottle	refrigerator
$NaHCO_3$ (e.g. Sigma S-5761)	0.35 g	TC cabinet[a]
Hepes (e.g. Sigma H-3375)	5.95 g	TC cabinet

[a]TC cabinet = Temperature controlled cabinet

Mix 199 Medium, HEPES, and $NaHCO_3$ with 0.95 liters milliQ H_20.

Using 10M NaOH, adjust pH to ~7.4 and bring volume to 1 litre.

Sterile-filter medium into bottles.

Store at 4°C for up to 3 months.

Date and label "OCM-Supplements".

Table H2. OCM + Supplements

Ingredient	Amount	Location
BSS + Heparin (Stock)	20 ml	freezer
Pen/Strep (e.g. Gibco 15140-122)	10 ml	freezer
L-Glutamine (Stock)	10 ml	freezer
OCM,- Supplements (Stock)	1 L	refrigerator

Day of use, add BSS + heparin, Pen/Strep and L-Glutamine.

Date and label "OCM + Supplements".

Make 500mL if only one person is slashing – half of Pen/Strep and L-Glutamine aliquots can be refrozen.

Table H3. 10x Saline Stock Solution

Ingredient	Amount	Location
NaCl	90 g	TC cabinet[a]
MilliQ H_2O	1000 ml	

[a]TC cabinet = Temperature controlled cabinet

Thoroughly mix ingredients.

Sterile-filter.

Date, label "10X Saline", and store at 4°C.

Table H4. 1X Transport Saline 0.9% (Prepare from 10X solution)

Ingredient	Amount	Location
10x Saline	100 ml	refrigerator
Pen/Strep (Gibco 15140-122	10 ml	freezer

Mix Ingredients and fill with MilliQ H_2O to 1 liter.

Date, label, and store at 4°C.

Table H5. Holding Medium (HM) – HEPES Talp

Ingredient	Amount	Location
BSA, Fraction V (Sigma A-3311)	120 mg	refrigerator
HEPES-TL	39.2 ml	refrigerator
Na Pyruvate (Sigma P-5280)	0.4 ml	refrigerator
Pen/Strep (Gibco 15140-122)	0.4 ml	freezer

pH should be = ~7.4.

Sterile-filter.

Date, label "HEPES-Talp" and store at 4°C for 1 week.

APPENDIX I. *IN VITRO* MATURATION OF BOVINE OOCYTES

1. Materials

- Petri dishes
- Mouth pipette (optional)
- Heat-pulled Pasteur pipette
- Oocyte Maturation Medium (OMM – See Table H1)
- Medical grade mineral oil

2. Preparation

1. Add 8.835 ml TCM, 20 µl FSH stock, 125 µl of LH stock, 1 ml of FBS and 100 µl of pen/strep to a 15 ml tube. Filter the medium (0.4 µm membrane). See Zhang *et al.,* 1992).
2. Add 10 µl estradiol stock
3. Prepare 35-µl maturation droplets of filtered medium under oil and equilibrate in incubator (5% CO_2) for at least 3 hours or add 250 µl of maturation medium to each well.
4. Prepare 75 µl wash droplets.

3. Procedure

1. Rinse the COC at least twice in the 75 µl droplets containing OMM.
2. Transfer 10 COC to each 25-µl droplets of OMM (or OMM2).
3. Incubate for 22 hours at 39°C and 5% CO_2 for IVF oocytes or incubate for 16 hours at 39°C and 5% CO_2 for NT oocytes.

It is essential the oocytes be collected, washed and incubated in OMM as quickly as possible to ensure maximum developmental rates. This entire process should never exceed 2 hours.

Additionally, blood is toxic to oocytes and embryos, so it is imperative that they be washed thoroughly to remove blood prior to transfer to OMM.

APPENDIX J. BOVINE IVF PROTOCOL

1. Materials:

- Centrifuge Carriers
- Percoll (ENHANCE-S Plus)
- Sperm-TL
- IVF Talp
- HEPES Talp
- SP Talp
- PHE
- Heparin
- 15 ml Conical Tubes
- Petri dishes
- Mouth pipette (optional)
- Heat-pulled Pasteur pipettes
- Sterile pipette tips and pipettor
- Microcentrifuge tubes (1.5 ml)
- Standard haemocytometer
- Centrifuge
- Stereomicroscope
- Medical grade mineral oil
- Laboratory tissues (e.g. Kimwipes)

2. Preparations:

a. Move PHE (400 µl) and Heparin (200 µl) from freezer to oven (39°C). PHE should be covered with aluminium foil (light sensitive).

b. Make fertilization microdrops

   i. Make five 44 µl drops of fertilization media (IVF-Talp) in each 35 mm dish. Cover with pre-warmed and pre-gassed mineral oil. (10 oocytes per drop).

   ii. Make four 70 µl drops of FM in a 35 mm dish (washing medium). Cover with mineral oil.

   iii. Equilibrate in CO_2 incubator (39°C) at least 2 hours.

c. Fill 1 conical tube with ~10 ml Hepes-Talp. Label tube.

d. Fill 1 conical tube with ~5 ml of IVF-Talp. Label tube.

e. Fill 1 conical tube with 5 ml SP-Talp. Label tube.

f. Also prepare 1 conical tube with 8mL Hepes-Talp.

g. Transfer tubes of HEPES-Talp (cap tightly) and SP-Talp (cap tightly) to the water bath (39°C).

h. Transfer IVF-Talp (cap loosely) to CO_2 incubator.

i. Prepare Percoll Gradient: Label 1 conical tube “Percoll Gradient” and fill the tube with 100 µl of Sperm-TL and 900 µl of ENHANCE-S Plus.

j. Carefully, transfer Percoll Gradient to the water bath.

3. Procedures:

a. At 22 to 24 hours post-maturation thaw 1 straw of semen in water at 39°C for 30 seconds. When getting semen straws out of the LN2 tank – make sure not to raise anything above the frost line. Use special semen forceps.

b. Dry a straw, hold it in a laboratory tissue to keep it warm and dark, cut the sealed ends off and slowly layer thawed semen on top of the Percoll Gradient Centrifuge at 1,200 rpm for 20 minutes.

c. Check viability of the thawed semen. Dilute one drop of semen with SP-Talp and place 5 µl of the suspension on a slide. View at 40X magnification to assure that motile sperm are present.

d. While centrifuge is running, pour 1 ml of HEPES-Talp (from conical tubes in CO_2 incubator) into Petri dish (35 mm). Remove oocytes from each well of OMM plate and transfer to the dish containing HEPES-Talp.

e. Transfer the oocytes to the washing medium.

f. Transfer up to 10 oocytes into each 44 µl fertilization drop or 425 µl in 4-well dish (IVF Talp, previously located in the incubator). Return IVF plate back to incubator when finished. *You only have 15 minutes to wash and transfer all oocytes to IVF 4-well plates. Set a timer and ask for help if necessary.

g. After centrifuge stops, carefully remove carrier with the Percoll gradient from the centrifuge. There should now be a sperm pellet, if not you must start completely over with new gradient and semen.

h. Within the laminar flow hood, aspirate the Percoll down to the sperm pellet. Slowly add the 5 ml of pre-warmed SP-Talp to the conical tube containing the sperm pellet. Transfer the tube to the second pre-warmed centrifuge carrier and centrifuge at 1200 rpm for an additional 10 minutes.

i. After the centrifuge stops, aspirate the SP-Talp down to the sperm pellet. Return the conical tube with the sperm pellet to the water bath.

j. Determine Sperm Pellet Concentration (see standard haemocytometer procedure)

   i. Gently swirl the sperm pellet to mix the sperm with any remaining medium. Use a clean pipette tip to transfer 5 µl of sperm into 95 µl of water, pipette gently to mix.

   ii. Clean the haemocytometer and coverslip by washing with water followed by 70% EtOH; dry with a tissue.

   iii. Using a new pipette tip, transfer 10 µl of diluted sperm into each chamber (each side) of the hemocytometer

   iv. Use 40X magnification to count sperm cells in the 5 squares arranged diagonally across the central square on one side of the hemocytometer. Use an event counter to keep track of how many cells are counted. Record the count.

   v. Continue counting on the second side of the hemocytometer counting 5 diagonally arranged squares to obtain the total hemocytometer count. If the count of one side varies more than 10% from the other side, then the diluted sample was not properly mixed. Repeat procedure starting at step I-1. When the count is consistent, record the total count and continue procedure.

   vi. Clean hemocytometer and coverslip with water followed by EtOH.

k. Preparing sperm suspension for insemination.
Note: The final sperm suspension used to IVF is composed of fertilization medium and sperm pellet produced by Percoll separation. The following the worksheet will simplify this procedure.

   i. Calculations are based on the following parameters:

- 300 µl of final sperm suspension will be prepared
- 1 x 10^6 sperm/ml is desired in the final fertilization medium (this concentration can be adjusted if needed using Step 3 below)

ii. Calculate the volume of sperm pellet needed per 300 µl of final sperm suspension using the formula:
- 7 500 / x = µl of sperm pellet to make 300 µl of final sperm suspension when inseminating with 1 x 10^6 sperm/ml
- When x is the average haemocytometer count (total haemocytometer count divided by 2)

iii. Adjust for desired sperm concentration (Step 3): If a concentration other than 1 x 10^6 sperm/ml is desired, then the volume of the sperm pellet must be adjusted to accommodate that difference. To adjust this volume perform the following calculation:
- Sperm concentration desired / 1 x 10^6 sperm/ml = sperm concentration adjustment factor
- Multiply the volume of the sperm pellet calculated in Step 2 by this adjustment factor to yield the volume sperm pellet needed to prepare 300 µl of final sperm suspension at the desired concentration.
- Example: If a bull requires 2 x 10^6 sperm/ml rather than 1 x 10^6 sperm/ml
  - x 10^6 sperm/ml / 1 x 10^6 sperm/ml = adjustment factor of 2
- (adjustment factor) x (µl of pellet needed for 1 x 10^6 sperm/ml) = µl of sperm pellet needed to yield 2 x 10^6 sperm/ml in 300 µl of final sperm suspension providing 2 x 10^6 sperm/ml in the fertilization drop.

iv. Calculate volume of fertilization medium needed in the final sperm suspension: Substract the volume found in Step 3 from 300 µl from Step 1.

v. Place the calculated amount of fertilization medium (Step 4) into and Eppendorf microcentrifuge tube. Then add the calculated amount of sperm pellet (Step 3) to the tube. Sperm stick to plastic, so add the fertilization medium to the tube first. Mix gently by pipetting up and down several times within the tube. Immediately begin fertilizing the drops since the pH of this solution will change rapidly.

l. Fertilization

i. Add 2 µl heparin (for a final concentration of 2 µg/ml of heparin in the fertilization medium), 2 µl of PHE and 2 µl of final sperm suspension to each drop. If 4-well dish was used, add 20 µl of heparin, 20 µl PHE and 20 µl of sperm.

ii. Record time and date on each fertilization dish.

iii. Incubate for 18 h at 39°C in a humidified atmosphere of 5% CO_2 in air.

m. Embryo Culture

i. Make five 20-µl drops of CR1aa (1-3 days) in a 35 mm dish. Cover the drops with oil. Equilibrate the medium for at least 20 min.

ii. Thaw one vial of hyaluronidase (1mg/ml). Place the solution in a 15 ml conical tube. Incubate the tube in the water bath for a couple min.

iii. Place the oocytes in the tube containing the hyaluronidase solution.

iv. Vortex the oocytes at maximum speed for 3 min.

v. Transfer the oocytes to one 35 mm dish containing Hepes-Talp.

vi. Wash the oocytes four times in CR1aa (1-3 days).

vii. Place 10 oocytes in each drop of CR1aa (1-3 dys).

viii. At day 3 transfer the oocytes in drops of CR1aa (3-7 days).

ix. Check development at day 7.

4. Media Preparation

a. Sperm - TL

Ingredient	Final (mM)	mg/100ml	Location
NaCl (Sigma S-5886)	100	582	TC cabinet[a]
KCl (Sigma P-5405)	3.1	23	TC cabinet
$NaHCO^3$ (Sigma S-5761)	25	209	TC cabinet
NaH_2PO_4 (Sigma S-5011)	0.29	3.48	TC cabinet
Hepes (Sigma H-3375)	10	238	TC cabinet
Lactic Acid (Sigma L-7900)	21.6	183.4 µl	refrigerator
Phenol Red (Sigma P-0290)	1 µl/ml	100 µl	TC cabinet
$CaCl_2•2H_20$ (Sigma C-7902)	2.1	29	desiccator
$MgCl_2•6H_20$ (Sigma M-2393)	1.5	31	desiccator

[a]TC cabinet = Temperature controlled cabinet

Add NaCl, KCl, $NaHCO_3$, NaH_2PO_4, HEPES, Lactic acid, and Phenol red into a beaker. Bring volume to 80 ml with ddH_2O and dissolve completely.

$CaCl_2•2H_2O$ and $MgCl_2•6H_2O$ should be dissolved in a small amount of double distilled (dd) H_2O before added to other ingredients.

Check for pH = ~7.4 and then adjust volume to 100 ml with ddH_2O.

Sterile-filter into a bottle.

Date, label "SP-TL", and store at 4°C for up to 2 weeks.

b. IVF-TL

Ingredient	Final (mM)	mg/100ml	Location
NaCl (Sigma S-5886)	114	666	TC cabinet[a]
KCl (Sigma P-5405)	3.2	23.5	TC cabinet
$NaHCO_3$ (Sigma S-5761)	25	210.4	TC cabinet
NaH_2PO_4 (Sigma S-5011)	0.34	4.08	TC cabinet
Lactic Acid (Sigma L-7900)	10	84.92 µl	refrigerator
Phenol Red (Sigma P-0290)	1 µl/ml	100 µl	TC cabinet
$CaCl_2•2H_20$ (Sigma C-7902)	2	30	desiccator
$MgCl_2•6H_20$ (Sigma M-2393)	0.5	10	desiccator

[a]TC cabinet = Temperature controlled cabinet

Add NaCl, KCl, NaHCO3, NaH2PO4, Lactic acid, and Phenol red into a beaker.

Bring volume to 80ml with ddH2O and dissolve completely.

CaCl2•2H2O and MgCl2•6H2O should be dissolved in a small amount of ddH2O before added to other ingredients.

Check for pH ~7.4 and then adjust volume to 100ml with ddH2O.

Sterile-filter into a bottle. Date, label "IVF-TL", and store at 4oC for 2 weeks.

c. HEPES-TL

Ingredient	Final (mM)	mg/500 ml	Location
NaCl (Sigma S-5886)	114	3330	TC cabinet[a]
KCl (Sigma P-5405)	3.2	120	TC cabinet
$NaHCO_3$ (Sigma S-5761)	2	84	TC cabinet
NaH_2PO_4 (Sigma S-5011)	0.34	20.4	TC cabinet
Hepes (Sigma H-4034)	10	1,200	TC cabinet
Lactic Acid (Sigma L-7900)	10	424.6 µl	refrigerator
Phenol Red (Sigma P-0290)	1 µl/ml	500 µl	TC cabinet
$CaCl_2•2H_20$ (Sigma C-7902)	2	150	desiccator
$MgCl_2•6H_20$ (Sigma M-2393)	0.5	50	desiccator

[a]TC cabinet = Temperature controlled cabinet

Add NaCl, KCl, $NaHCO_3$, NaH_2PO_4, Hepes, Lactic acid, and Phenol red into a beaker.
Bring volume to 450 ml with ddH_2O and dissolve completely.
$CaCl_2•2H_2O$ and $MgCl_2•6H_2O$ should be dissolved in a small amount of ddH_2O before added to other ingredients.
Check for pH ~7.4 and then adjust volume to 500 ml with double distilled H_2O.
Sterile-filter into a bottle. Date, label "HEPES-TL", and store at 4°C for 2 weeks.

d. IVF-Talp

Ingredient	Amount	Location
BSA, EFAF (Sigma A-6003)	60 mg	refrigerator
IVF-TL	9.8 ml	refrigerator
Na Pyruvate (Sigma P-5280)	100 µl	refrigerator
Pen/Strep (Gibco 15140-122)	100 µl	freezer

pH should be ~7.4. Sterile-filter. Date, label "IVF-Talp", and store at 4°C for 1 week.

e. HEPES-Talp

Ingredient	Amount	Location
BSA, Fraction V (Sigma A-4503)	60 mg	refrigerator
HEPES-TL	20 ml	refrigerator
Na Pyruvate (Sigma P-4562)	0.2 ml	refrigerator
Pen/Strep (Gibco 15140-122)	0.2 ml	freezer

pH should be ~7.4. Sterile-filter. Date, label "HEPES-Talp", and store at 4°C for one week.

f. Sperm – Talp (SP-Talp)

Ingredient	Amount	Location
BSA, Fraction V (Sigma A-4503)	60 mg	refrigerator
SP-TL (Specialty Medium BSS-009-D)	9.5 ml	refrigerator
Na Pyruvate (20mM Stock)	0.5 ml	refrigerator
Pen/Strep (Gibco 15140-122)	100 µl	freezer

pH should be ~7.4. Sterile-filter. Date, label "SP-Talp", and store at 4°C for one week.

g. 10X SP-TL (for Percoll gradient)

Ingredient	g/100 ml	Location
NaCl (Sigma P-5886)	4.675	TC cabinet[a]
KCl (Sigma P-5405)	0.23	TC cabinet
NaH_2PO_4 (Sigma S-5011)	0.348	TC cabinet
HEPES (Sigma H-4034	2.38	TC cabinet

[a]TC cabinet = Temperature controlled cabinet

DO NOT adjust pH.

Bring volume to 100 ml with double distilled H_2O.

Sterile filter. Date, label "10X SP-TL", and store at 4°C one month.

APPENDIX K. HARVESTING OF TISSUE SAMPLES FOR CRYOCONSERVATION OF SOMATIC CELLS

Collection of somatic cells for gene banking can be undertaken in either of two approaches, depending on the situation in a given country, 1) collection and cryopreservation in the field and 2) field collection with processing and freezing in the laboratory. Protocols for both approaches are described below.

In the field (same for both protocols):

1) To prevent the infection of tissue samples, ensure that the conditions for sampling are as clean as possible, especially the working area for placement of sampling equipment.
2) Identify the animals to be sampled.
3) Restrain each animal and record as much of the following information as possible (see Chapter 9 for more details about data collection):
   - site-specific data, including GPS coordinates,
   - animal-specific information (Chapter 10), including a digital photo,
   - sample specific information (e.g. vial and animal numbers),
4) Clean and disinfect the area of skin from where the sample is to be taken.

Protocol 1 – One-step collection and freezing in the field (Groeneveld, 2008)

In the laboratory prior to field collection:

1) Prepare a cryoprotectant medium by mixing 70% PBS and 30% of 87% glycerol, yielding a final concentration of 26.1% glycerol.
2) Store aliquots of the mixture in small, tightly-capped vials (2–3ml, e.g. centrifuge tubes) sufficient for 5-6 tissue samples (0.3ml per sample).

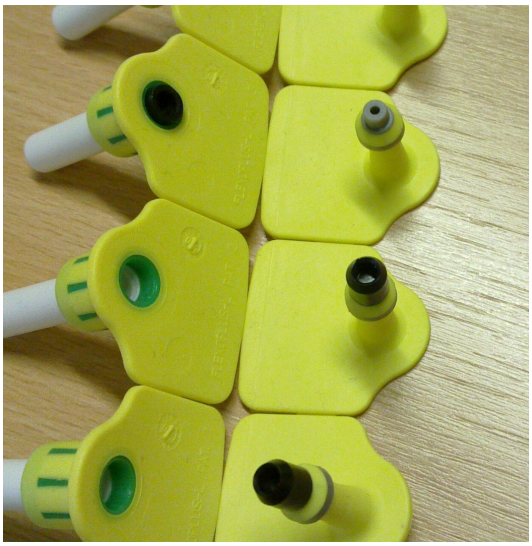
In the field:

Required equipment include:

- specialized ear tagger with tissue biopsy punch (See Figure J1),
- combination ear tag / sample cap (see Figure J2),
- portable cryotank with liquid nitrogen,
- micropipette and tips,
- plastic storage vials with cryoprotectant (prepared as described above)
- latex gloves, disinfectant spray (e.g. Ethanol 70%) and animal restraining equipment

Figure J1. Ear tagger

Figure J2. Ear tag / sample vials



Collection procedure:

1) Fill the sample cap of the ear tag with 0.3ml of the cryoprotective medium and secure it in position in the eartagger.
2) Place the ear tagger[22] in the proper position on the ear of the animal and collect the sample by squeezing firmly the handles of the ear tagger. This will push the ear biopsy into the sample cap.
3) Remove the sample cap containing the ear biopsy from the tagger and shake it gently to ensure the cryoprotectant medium fully covers the ear biopsy stored inside.
4) Place the the sample cap directly into the portable cryotank.
5) The samples may be moved to a larger tank for long-term storage upon return to the gene bank. This must be done as quickly as possible to avoid the risk of thawing.

Protocol Two – Field collection and laboratory processing

Required equipment include:

- Sampling tool (e.g. surgery kit, scalpel, hole punch or ear notcher/tagger)
- tweezers
- sample vials
- laboratory gloves, disinfectant spray (e.g. Ethanol 70%) and animal restraining equipment
- transport container with ice (e.g. Styrofoam box)

Collection procedure:

1) Use the chosen tool to obtain a skin sample (e.g. ear notch, skin biopsy, etc.).
2) Insert the sample into a sterilized, labelled storage vial.
3) Place the vial in the transport container.
4) Return to the laboratory for further processing and long-term storage.

Processing procedure:

1) Slice the tissue sample into small pieces preferably 0.25 to 1.0 cubic mm
2) Submerge in in phosphate-buffered saline (PBS) supplemented with 80 mg/ml of streptomycin sulfate, 60,000 units/ml of benzylpenicillin and 20,000 IU/ml of potassium penicillin G.
3) Wash the samples (4 to 5 pieces) in 20% fetal calf serum-PBS (FPBS)
4) Placed pieces in vitrification solution (20% [v:v] ethylene glycol, 20% [v:v] dimethylsufoxide in FPBS)
5) Load the mixture into 0.25-ml French straws (1.5 cm of FPBS, air bubble, samples in 4 cm of vs. air bubble and 1.5 cm of FPBS)
6) Seal the straws
7) Plunge the straws vertically into LN2.

Thawing of samples (both protocols):

Samples are thawed by holding the straw or sample cap in LN2 vapor for 10 seconds and then submerging them in a 23ºC water bath for 5 seconds. The sample is then expelled into a dish, diluted with 0.25 M sucrose in FPBS for 5 minutes and then placed in FPBS (without sucrose) for 5 minutes. Samples can then be cultured for SCNT or used for DNA extraction.

[22] Eartaggers and similar sampling tools can be purchased commercially from various sources, including the following: www.prionics.com, www.caisley.de, www.biopsytec.com and www.prosampler.com.

APPENDIX L. COLLECTION AND FREEZING OF POULTRY GONADAL TISSUE

Storage of gonadal tissue is a recently-adopted procedure for cryoconservation of special research lines of poultry at the public research institutes in Canada. The cryoconservation procedure is outlined below (adapted from Silversides *et al.,* 2008).

Collection of Ovarian Tissue

1) Euthanize female chicks and open the abdominal cavity.
2) Locate the ovary, which can be recognized as an irregularly shaped structure attached to the dorsal wall on the left side of the abdominal cavity.
3) Detach the ovary by using a fine forceps or tweezers.
4) Remove the connective tissue from around the ovary and dissect the cortical part.
5) Cut the ovary into two or three pieces.

Collection of Testicular Tissue

1) Euthanize male chicks and open the abdominal cavity.
2) Locate the testes, which are worm-like organs on both sides of the dorsal part of the abdomen.
3) Detach the testes by using a fine forceps or tweezers.
4) Remove any connective tissue.
5) Cut each testes into four to five pieces (each about 1.0 to 1.5 mm^3)

Tissue Cryopreservation (same for both testes and ovaries)

1) Upon removal, place gonads in solution of 1.0 mL of phosphate buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 L H_2O @ pH 7.4) and keep on ice until freezing (within 4 hours).
2) For freezing, prepare a cryoprotectant solution of Dulbecco's Modified Eagle's Medium (Sigma Chemical Co., St. Louis, MO) containing 10% (v:v) dimethylsulfoxide and 10% (v:v) fetal bovine serum.
3) Use the fine forceps to transfer the tissue into a 0.5 ml cryo straw.
4) Inject the cryoprotectant into the straw by using a long needle.
5) Eliminate bubbles within the straw and seal.
6) Allow the temperature of the straw contents to equilibrate by maintaining them at room temperature for 20 minutes, periodically inverting the straws 3 to 5 times.
7) Freeze the straws according to the following programme:
   - Room temperate to 10°C at -1°C/min
   - 10°C to -7°C at -0.5°C/min
   - Held at -7°C for 10 min
   - Ice nucleation with a forceps cooled in LN2
   - -7°C to -55°C at -0.5°C/min
8) Plunge the straws into liquid nitrogen.

APPENDIX M. REFERENCES FOR APPENDICES

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