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DRAFT GUIDELINES FOR THE CRYOCONSERVATION OF ANIMAL GENETIC RESOURCES

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DRAFT GUIDELINES FOR THE CRYOCONSERVATION OF ANIMAL GENETIC RESOURCES

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 germplasm 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* 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 genetics resources.

To formally recognize and address these problems, the Member States of the FAO developed the *Global Plan of Action for Animal Genetic Resources*, 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 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,* 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 on In Vivo Conservation of Animal Genetic Resources* and this document, *Guidelines on Cryoconservation of Animal Genetic Resources.*

The development and operation of a gene bank for cryoconservation of animal genetics 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 genetics resources.

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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, and 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 Focal Point for Animal Genetics 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.

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THE GOAL AND STRUCTURE OF THESE GUIDELINES

The objective of these Guidelines is to provide technical guidance and to serve as decision aid among the available cryoconservation options, and 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 for 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 cryoconservation 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. Biological material undergoes a number of notable and sometimes drastic changes when subject to cryopreservation.

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 precise and detailed 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.

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.

Chapter 11 describes reproductive methods to be employed for the eventual utilization of the stored germplasm.

Chapter 12 addresses the legal issues of cryoconservation, which are of importance because although animal genetic resources can be considered a public good, the animals that will be the source of the germplasm are usually privately owned.

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 13.

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 germ plasm.

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
mg: Milligram
ml: Milliliter
MTA: Material Transfer Agreement
NT: Nuclear Transfer (a scientific term used for cloning)
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
SCC: Semen Collection Center
SCNT: Somatic Cell Nuclear Transfer
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 this is primarily the active breeding of animal populations for food and agricultural production such that diversity is best 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* and *Guidelines for In Vivo Conservation of Animal Genetic Resources*.

1.2. *Ex situ* conservation

In the context of domestic animal diversity this means conservation away from the habitat and production systems that developed the resource. This will include both storage as live animals away from the habitat and cryoconservation.

1.2.1. Ex situ - in vivo *conservation*

This 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 finance and commitment is available to maintain generations of animals to the standards required for 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

This 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 (CBD, 1992, article 8) emphasizes the importance of *in situ* conservation and considers *ex situ* conservation as an essential complementary activity to *in situ* (CBD, 1992, article 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 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 Animal Genetics Resources, 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 AnGR, if any, cryoconservation programmes should be undertaken.

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

	Technique		
Objective	**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	-	+	++

References

CBD, 1992.

Gustavo Gandini and Kor Oldenbroek. Strategies for moving from conservation to utilisation. Chapter 2. In: Utilisation and conservation of farm animal genetic resources. Kor Oldenbroek (ed.). ISBN: 978-90-8686-032-6. First published, 2007. Wageningen Academic Publishers The Netherlands, 2007.

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 animal genetic resources. 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 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 countries 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 extisting 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 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. In other situation, 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.

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 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 NASP. 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, artificial insemination 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 developing NASP, 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 12).

2.3.4. The National Coordinator for AnGR

The FAO National Coordinator (NC) for AnGR will be an important partner 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 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.

2.4.1. Funding from international agencies

There are two key features that animal conservation projects should aim to provide in order to attract funding from international agencies:

- The project needs to be part of a national strategy for conservation of the whole environment taking account of the ecosystem including plants and forests, since 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 has 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 are 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 genetic resources 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 reducing inbreeding levels and broadening breed diversity in the event of a bottleneck or to backup the industry when particular selection strategies are deemed, at a later time, as not appropriate.

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 time 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 conservation of small populations (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 would occur. Decrease of effective population size and the resulting high level of inbreeding can lead to an increased relative frequency of deleterious alleles that were not apparent in a larger population. 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 effective size of the gene pool is still very small. Gene bank resources may be needed to remove deleterious genes from the population by introducing new genotypes (e.g. semen doses) from the original (larger) population.

A fourth important use of the cryopreserved genetic resources 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 modify or reorient the genetic trend of a selected population. Verrier *et al.,* (2003) suggested the storage of original and extreme genotypes that have extreme breeding values for specific traits, that carry rare alleles, or that represent specific founders or pedigree lines. A specific example can be the storage of material from traditionally dual-purpose cattle, reoriented to beef or milk production.

Finally gene banks can serve as the primary source of material for country researchers performing DNA research. This type of service activity 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 are of 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 categories 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 *effective population size* (N_e) of 50 head (See Box 3.1), so that the rate of inbreeding can be held at 1% per generation (FAO, 1998). However, Meuwissen and Woolliams (1994) showed that the recommended effective population size 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 50 males a repository has a 63% chance of capturing alleles with a frequency of 0.01. This later 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 typically 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 targeted number of animals will slow or limit the ability to meet collection goals.

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 establish how the germplasm would be used, reconstitution being the most important. Different mating strategies exist across species and whether semen or embryos are used in the reconstitution process. Furthermore, collection goals are heavily influenced by 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.

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, which is usually smaller than the absolute population size.

The effective population size (N_e) is the number of breeding individuals in an idealized population that would show the same amount of disperse on of allele frequencies under random genetic drift or the same amount of inbreeding as the population under consideration. 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 by 2 males and 100 females.

A 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 effective population size. 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.

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 effective population size is 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.

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 embryos or somatic cells (FAO, 1998). If less than this number is available then the animals are selected irrespective of the relationships among them. More than 25 is, of course, beneficial if resources permit, although in some cases more animals does not necessarily mean more genetic variation 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 Genetics 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.

According to ERFP (2003), a minimum number of 25 donors of semen are suggested. 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 lower this number, but this will decrease genetic variation stored. In such cases, it is recommended to 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 only 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 will 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 worse case scenarios where an entire breed could be 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, elimination of deleterious genes or accessing gnees and gene combinations that may be useful when selection goals change. 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 then 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.

- Livestock breeding populations have often been selected for one specific trait at the exclusion of others and as a result, the population lacks the genetic variation required to effectively alter phenotypes to adjust to new market conditions (e.g., changes in the value of fat in milk). 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 which assist in managing the collection.

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 poises 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 people

raising the breed in question. 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 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 component of an overall collection serves a role of providing ready access to relevant germplasm for a) potential development of research populations of animals, b) a source of unique germplasm for industry if a breeding organization should need to change selection goals or eliminate a deleterious allele or c) the development of a new breed.

3.3.4. Evaluation Collection

There is a need to quantify the success the of the cryopreservation process from a sampled animal for the gene bank, particularly for semen. This portion of the collection allows for that type of use, 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.

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 which 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 germplasm that is no longer actively needed to the Core Collection.
2. Determining when quantities of germplasm exceed requirements for the Core animal and can be moved to the Working Collection.
3. Locating and obtaining samples from animals of interest for 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 or accept samples that no longer have commercial value to them.

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-accessioned. The Working Collection has the potential to be the most dynamic element of a breed's germplasm collection. Therefore, a major consideration in determining the size of the Working Collection is the additional space in the liquid nitrogen tank, the number of liquid nitrogen tanks the facility can accommodate, and the recurrent financial requirements.

Given these considerations it would seem reasonable to expect breed level Working Collections to 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 plant varieties. A similar approach is

envisioned for livestock and would also include acquiring samples from animals where interesting mutations may be evident. In addition, to adding new germplasm samples to the repository there is also 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-accessioning is a difficult undertaking and a gene bank needs a well established protocol before initiating this process. A set of potential reasons for de-accessing samples includes:

4. Genetics of the sample are too closely related to other samples in the collection (e.g. half-sibs or closer).
5. Sample post-thaw quality is low and similar genetics are in the repository.
6. 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, ovaries, 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 is 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 can be accomplished in a single generation.
- **Oocytes** may be considered for storage in gene banks along with semen, but techniques for freezing and thawing still need to be fine tuned and fielded evaluated. The advantage of oocytes is that through *in vitro* fertilization (IVF) desired matings can be selected.
- **Ovaries** Cryopreservation of ovaries could be another way to preserve the genotype of animals. Cryopreserved ovaries would be used as a source of oocytes.
- **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 Genetics Resources* for more information.
- **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 programmes exist in many countries, which could facilitate collection and future use of semen. In some cases, however, semen collection can be a problem, for instance when the animals cannot be trained for semen collection. 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 between 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.

Relative to other types of germplasm, semen has the disadvantage, however, that only a single complement of chromosomes is preserved. If only semen is conserved and the cryoconserved breed was completely lost, then another breed would need to provide the founder females for insemination (or the female gamete in the case of other reproductive techniques). A series of backcrossings would 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" (See Wakeland *et al.,* 1997 or Hospital *et al.,* 1992). 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.

4.2. Embryos

In cattle, cryopreservation of embryos has been successful and is a routine procedure. Both slow-freezing and vitrification protocols 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. The possibilities for cryopreservation of sheep embryos are similar to that for cattle (e.g. Fogarty *et al.,* 2000). Cryopreservation of pig embryos has long been quite problematic, due to extreme chilling sensitivity and the high lipid content of the pig embryos. However, recent studies have focused on overcoming particularly these problems and produced successful methods for cryopreservation of pig embryos (e.g. Nagashima *et al.,* 1999; Vajta, 2000) and live piglets have been obtained (e.g. Dobrinsky *et al.,* 2000; Nagashima 2007). Other farm animal species for which live offspring have been obtained from cryopreserved embryos include the horse (Ulrich and Nowshari, 2002), goat (Rodriguez Dorta *et al.,* (2007), dromedary camel (Nowshari *et al.,* 2005), and rabbit (Naik *et al.,* 2005). Pregnancies have been reported in Ilama (Aller *et al.,* 2002), and Red deer (Soler *et al.,* 2007).

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 genotype when needed. Embryo technology, however, is usually more costly and requires greater technical capacity than gene banking with semen.

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 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.,* Theriogenology 1994), 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.

4.4. Ovaries

Cryopreservation of ovaries could be another way to conserve the genotype of animals. 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 (transgenic and other) 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). 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). Further improvements are needed in the cryopreservation procedure and the grafting technique. For *ex situ* conservation of genetic diversity of farm animals, cryopreservation of ovaries or ovarian tissue may be less efficient than cryopreservation of embryos. 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.

4.5. 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. The opposite is true, however, for using the material when needed. Utilization involves 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, ET into recipients of the same species. The use of nuclear transfer means that the original mitochondrial genotype is lost.

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 breed reconstitution, 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 in the future. Therefore cryobanking of ear or other skin tissue can be considered as a cheap method of conserving genotypes for the more distant future, possibly even now for pigs.

4.6. 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 in vitro and in vivo approaches.

4.7. 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 breeding 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", one would like to have a cheap and fast collection of as many species and breeds as possible. Wherever financial resources and existing expertise and facilities are available, embryos are probably the best choice, but in lack of that, collection and cryopreservation of somatic cells could be considered as a possibility (Groeneveld *et al.,* 2008). Table 4.1 summarizes the characteristics and advantages and disadvantages of cryoconservation via different types of germplasm.

Table 4.1. 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	Depends on expected future efficiency of cloning
Backcrossing needed	Yes	No[3]	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 4 generations	IVM/IVF[4] surgical or nonsurgical ET	Surgical or nonsurgical ET	Transfer in enucleated oocytes, surgical or nonsurgical ET
Possible?	Yes	Yes	Yes	Low efficiency and many risks. Future development is likely!

[1] See Chapter 6 for more specific details.

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

[3] Yes, if only oocytes are stored.

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

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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 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 often 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 may also require the employment of an architect and 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, superovulation of 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 OIE standards for export, 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

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.

5.1.1.3. Ventilation

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.

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

Animal pens should be constructed with three factors in mind;

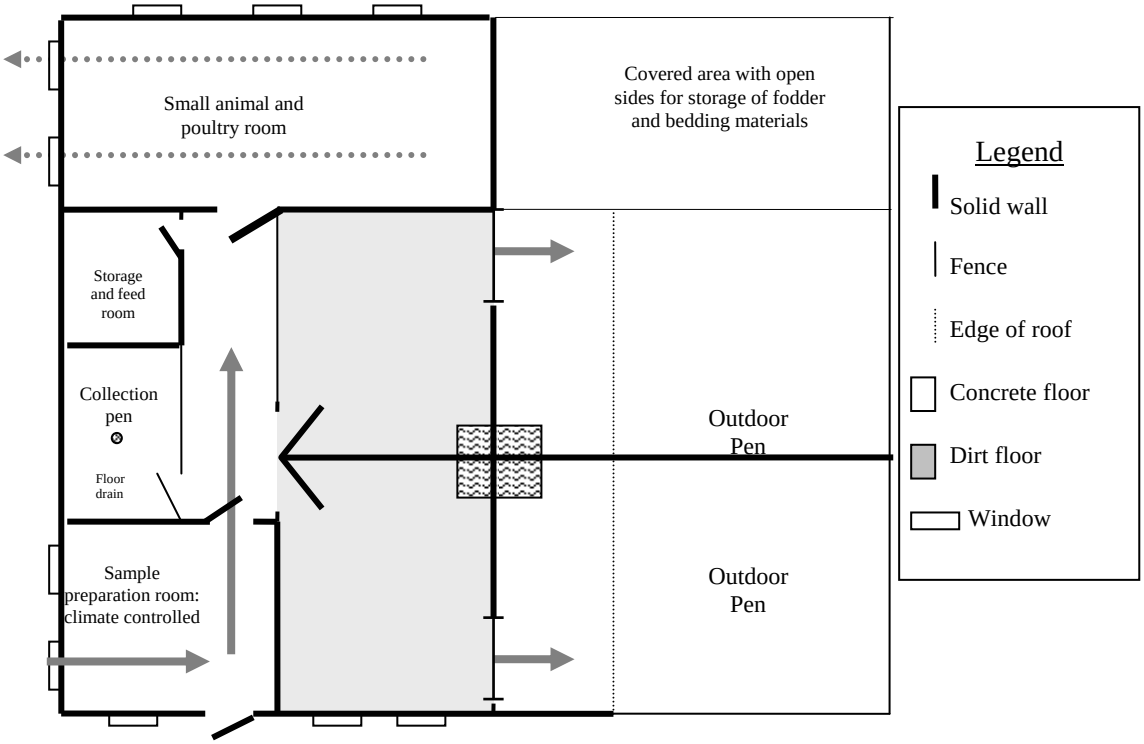
1. Size: The pens should be appropriate for the animals' size and be large enough so that minimal handling of animals is required when moving them into and out of the facility. For example, if sheep or goats are kept in pens that were designed for cattle then a large amount of time could be spent on 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. Materials: High-quality, durable pens should be constructed so that they can handle the repeated stresses of animals.
3. 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.

5.1.1.5. 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 four to eight rams and 20 to 30 roosters, and for collecting and processing semen samples and embryos or other genetic materials. The facility is equipped with indoor and outdoor pens 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 poultry 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 the environmental temperature controlled to decrease the potential of cold-shocking semen samples.

The airflow of the facility is also illustrated with the dotted and solid grey lines. The dotted line illustrates the airflow within the poultry room. The poultry room is completely separate from the remainder of the facility to minimize the spread of chicken byproducts (scratch, feces, 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.

Figure *5* .1. An example of the animal collection facility.



Total Area ~225 m^2

5.1.2. Germplasm processing and freezing laboratories

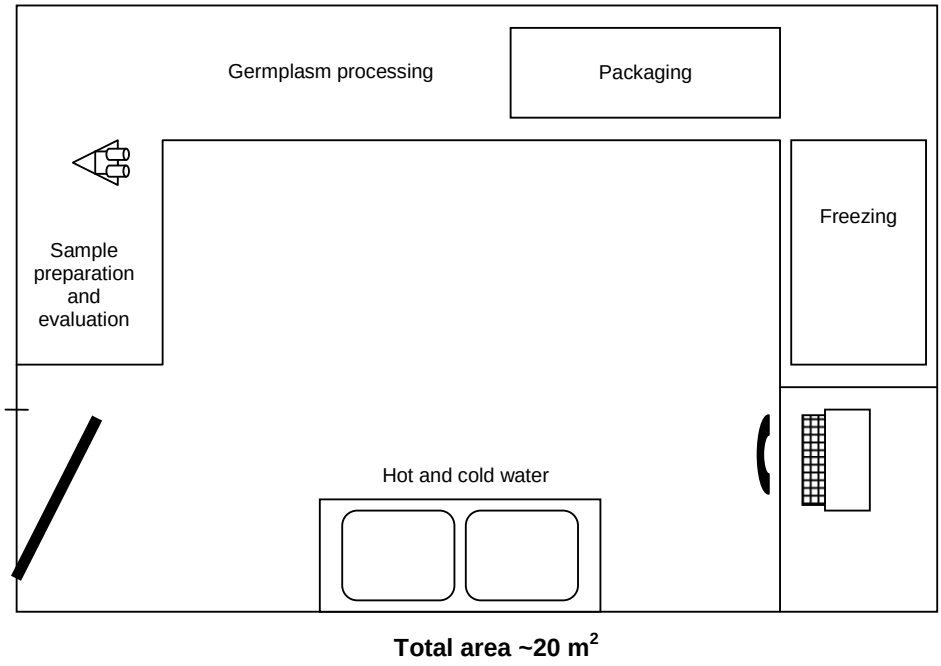
Although the processing and freezing laboratories may be part of the same physical structure as the animal collection and holding facilities, a distinct physical barrier between the two areas must be present, as the processing and freezing area must have a greater level of sanitation. The laboratory 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 more 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.



5.1.3. 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 liquid nitrogen (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.

Liquid nitrogen 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. Liquid nitrogen 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 ore 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 vapor 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 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 samples to 5°C:
  - 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 cryopreserve semen to levels in the thousands of straws.

- Wet lab 30 to 60 m^2
- Cryotank storage room 25 to 50 m^2 (approximately 400 000 to 600 000 straws)

- Ability to cool samples to 5° C:
  - Cold room (10 m^2), or
  - Cooler cabinet (2 m^2), or
  - styrofoam box (0.6 m^2)
- 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 computed assisted sperm analysis (CASA) unit and flow cytometers.

- Wet lab greater than 60 m^2
- Cryotank storage room greater than 50 m^2 (>500,000 straws)
- Ability to cool samples to 5°C:
  - Cold room (10 m^2 sq ft), or
  - Cooler cabinet (2 m^2), or
  - styrofoam box (0.6 m^2)
- Office for database management (20 m^2)
- Other office space (50 m^2)
- Capacity to store excess LN_2 or even to make LN_2.

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. Better quality tanks will last longer and usually use less LN2.

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 liquid nitrogen 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

- Microscope (phase-contrast – semen; stereo scope – embryos)
- Centrifuge
- Cooling samples (styrofoam or plastic cooler, cooler cabnet)
- Osmometer
- pH meter
- Method for determining sperm concentration (one or more of the following):
  - Spectrophotometer (fixed or portable)
  - Makler counter chamber (or desposable counting chamber), or
  - Hemocytometer
- Temperature measuring devices
- Dry LN2 shipping tanks
- Long term storage tanks
- Stereomicroscope for embryo evaluation (optional)

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 2010 prices).

	Small			
Item	Necessary	Optional	Medium	Large
Long term LN_2 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

Along with facility security there is a need for germplasm programs to develop continuity of operation plans that will facilitate the implementation of redundant systems in the event of a major problem. Such plans might include:

- Identifying a limited number of staff responsible to insure the facility's continuity of operations.
- Developing agreements with necessary suppliers (or alternative suppliers) for needed services in the event a serious need.
- Development of plans to move the collection in the event of civil unrest or other emergencies that threaten the safety of the collection.

Another possibility that merits strong consideration is the maintenance of a second site for storage of cryoconserved germplasm for insurance against at catastrophic event occurring to destroy the primary gene bank. 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.

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. 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. Collection efforts can be enhanced if the gene bank is located near to major roads or airport hubs within the country.

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 be 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.

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.
- 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 endeavor 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 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 capacity include both the accessibility of the animals for collection and the technical and financial capacities of the gene bank for 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 the population 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 prioritization of AnGR for conservation are presented in the *FAO Guidelines for In Vivo Conservation of Animal Genetics 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 a committee of expert stakeholders. Nevertheless, in certain situations gene bank managers and curators should have the flexibility to be preemptive 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.

6.1.2 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 not often 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 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 National Strategy and Action Plan for AnGR as well as a standard procedure in reporting on implementation of the Global Plan of Action for AnGR. FAO has produced *Guidelines on Surveying and Monitoring of Animal Genetic Resources* to assist countries in this endeavor. 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 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 behavioral, 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.

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 importance 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 Genetics 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 Genetics 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.

6.2. Collection targets for reconstituting populations

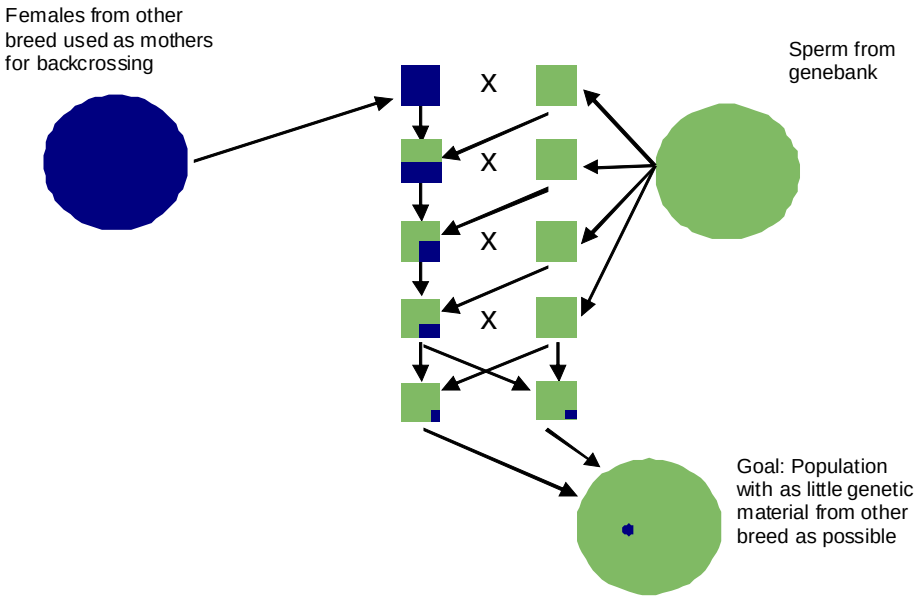
Once the decision has been taken on the particular breeds and populations to be collected for the gene bank and the type of germplasm to be collected the next step is to determine the types and amount of germplasm to be collected. These quantities will vary depending on the conservation

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

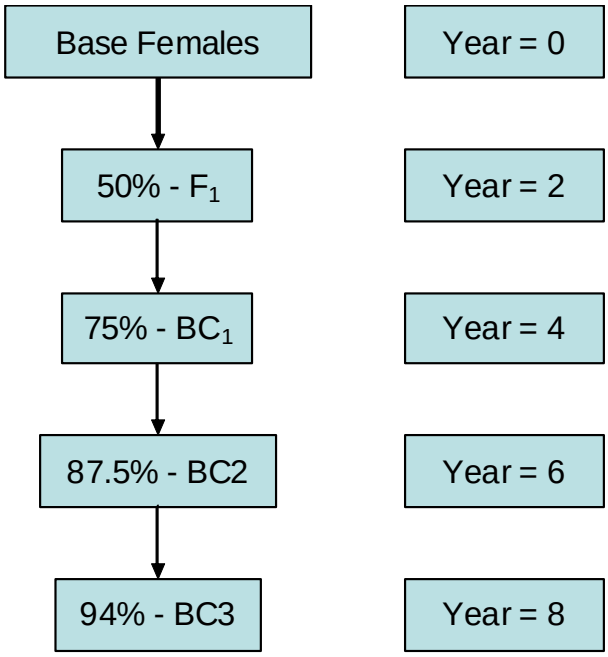
When semen is cryopreserved, the principal method for reconstituting a breed or population is through backcrossing (Figure 6.1). One starts with females of a common breed and with four backcrosses it is possible to reconstitute over 90% (4 generations ~ 94%; 5 generations = ~97%) of the conserved breed's 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.

Figure 6.1. Demonstration of population reconstitution with cryoconserved semen.



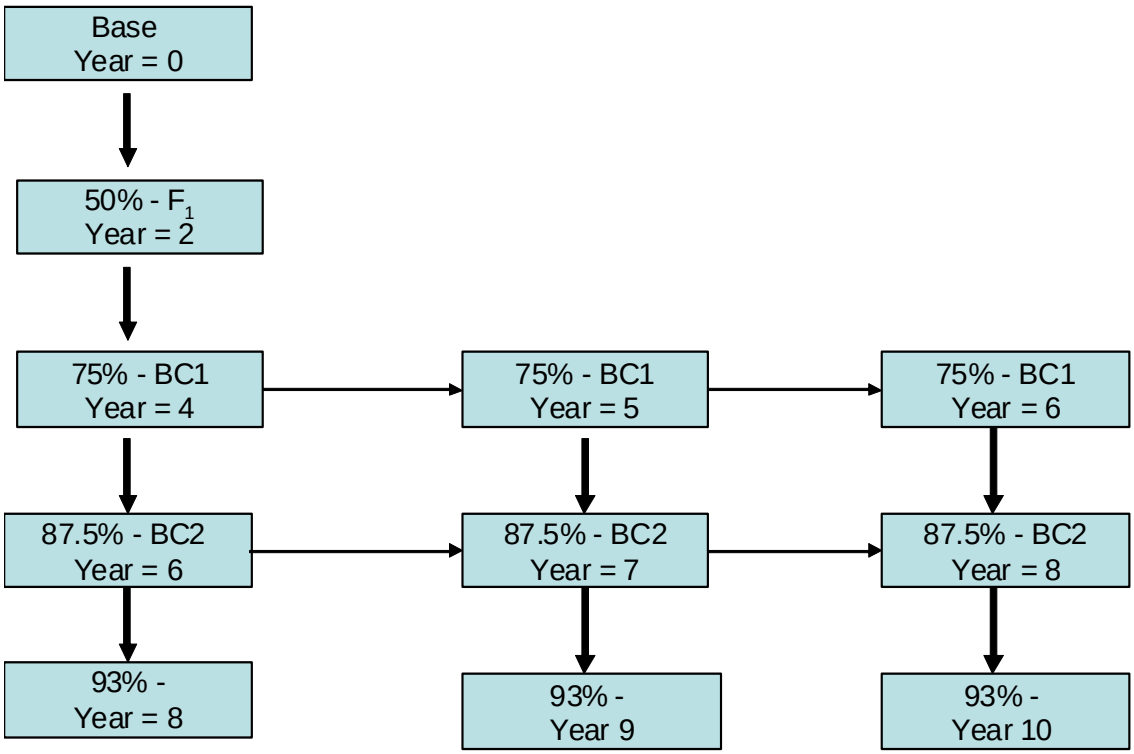
The length of reproductive cycles and conception rates impact the speed and efficiency with which populations can be reconstituted. To better establish collection goals, a view on how the cryopreserved material will be used to reconstitute a breed needs to be quantified. Previous reports (FAO, 1998; 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 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 effective population size.) For a number of conception rates and initial number of animals bred, the effective population size is below 50, the recognized target. However, this deficiency can be managed by having extra males 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 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		
	Bulls (N)			Bulls (N)			Bulls (N)			Bulls (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 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, especially 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 semen[1] 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	Pregnancy rate		
(N)	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.

	Pregnancy rate								
Founder	0.4			0.5			0.6		
females	Donor males (N)			Donor males (N)			Donor males (N)		
(N)	25	50	100	25	50	100	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 offsprping. 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

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, 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 for 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 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).

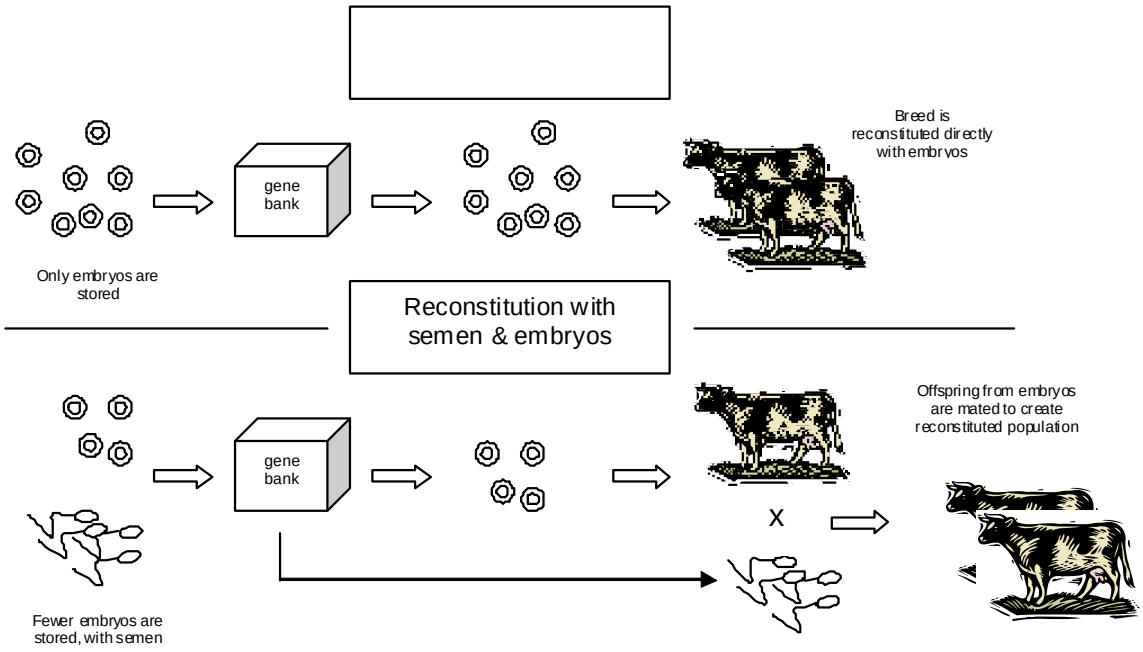
In such cases where sampling of 25 donors is not possible (or simply to economize), another option that may be considered is a hybrid scheme in which one stores a combination of semen and embryos (Boettcher *et al.,* 2005). In this strategy, smaller numbers of embryos are required than listed in Table 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 possibly 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. 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 effective population size 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.

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 6.1. 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 effective population size than represented by the living population only.

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 effective population size, a cryo-aided live scheme can be very beneficial, mainly because it will result in prolonged generation intervals and therefore a larger effective population size. Intensely selected breeds can actually have a small effective population size, 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

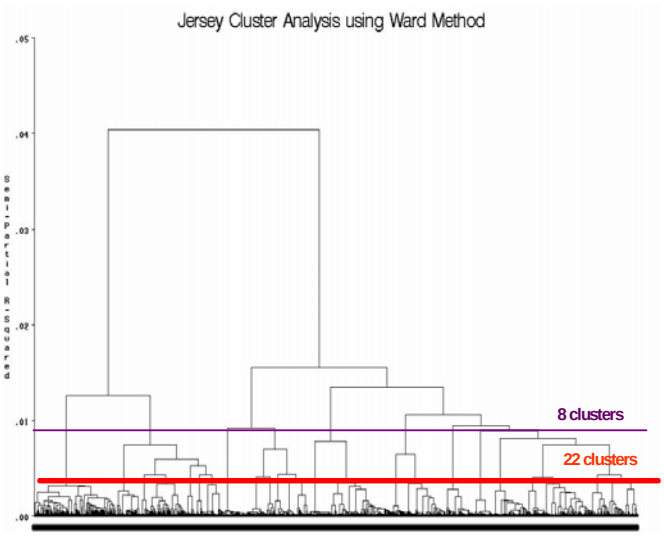
Here the major consideration is to select animals that have the least genetic relationships to one another. Several avenues exist depending upon existing information and resources available to make choices.

- 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 unique 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.

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

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 within the cluster
No. in Rep. = number of bulls selected for the gene bank from each cluster

- 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.

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).

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.

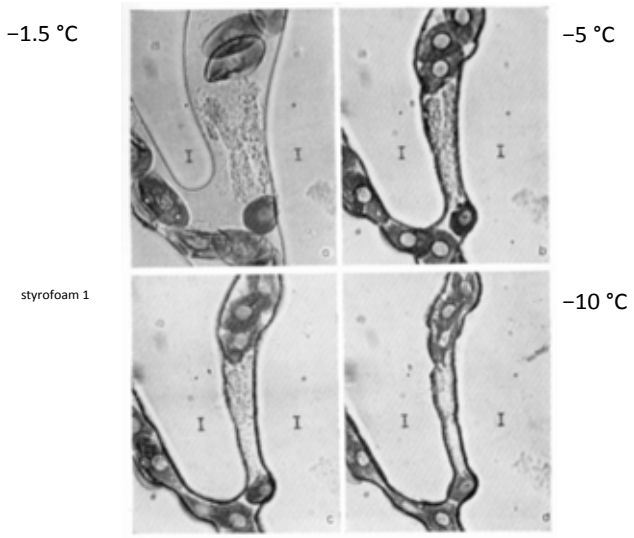
7.1. Slow freezing and vitrification

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. 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.

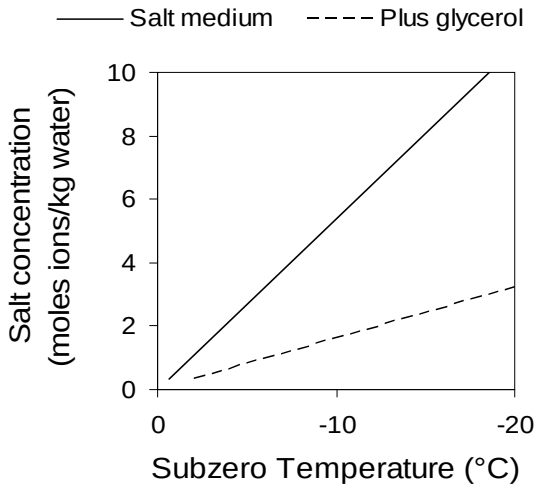
7.2.3. Conditions in the unfrozen fraction

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

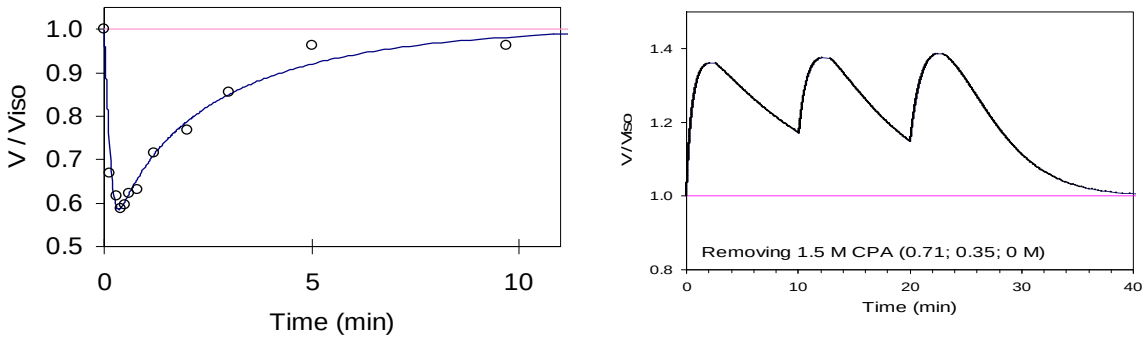
substantial initial glycerol concentration in the medium means that part of the extracellular and intracellular water is replaced by the glycerol. Hence, the amount of ice formed is lower, the unfrozen fraction remains larger, the degree of shrinkage of the cells is limited, and the electrolyte concentration 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 reswell 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 stronger (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).

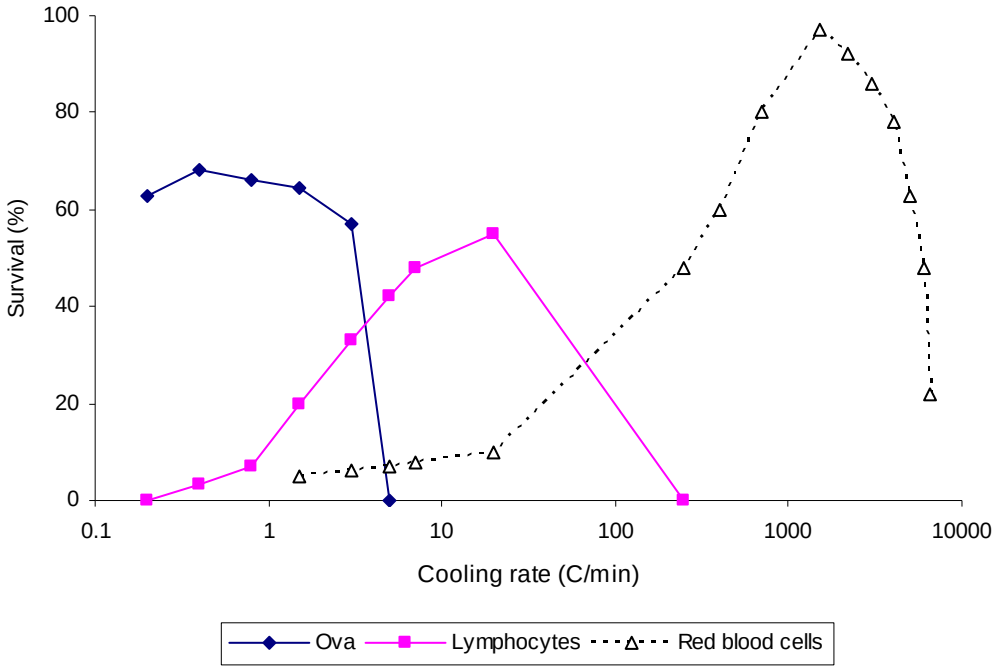
Ice growth is a rapid process, but transport of water through the cell membrane is a relatively slow process, because the membrane poses a resistance barrier. Therefore, as cooling and extracellular ice growth continue, the liquid water of the unfrozen fraction remains very close to equilibrium with the ice, but the intracellular water lags behind. This means that the water 'concentration' (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.

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 completely different due to the generation of heat of fusion. (Figure 7.6.)

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

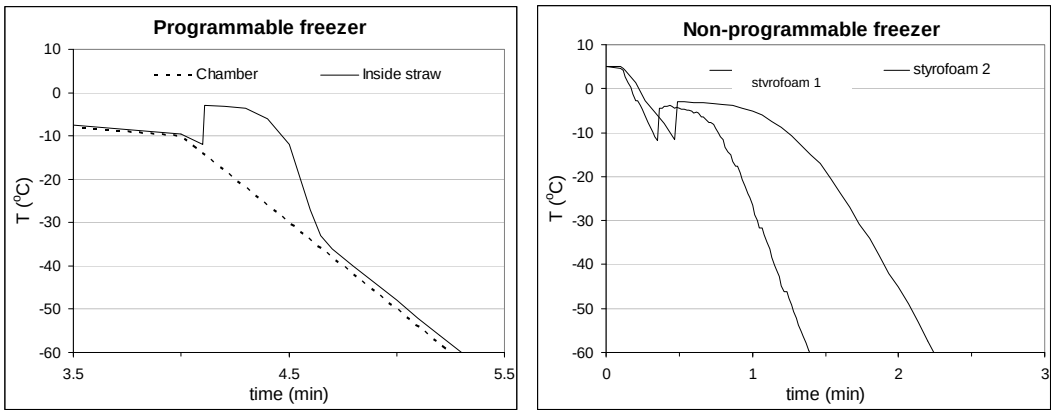
In non programmable freezers, the straws may be cooled by being exposed to vapour (or a cold surface) at a constant low temperature. An example of a simple system is the freezing of straws placed on a rack in a styrofoam box partially filled with LN2 without ventilation. The height of the straws above 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 set up. 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 cryoprotectants. 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 cryo-protective agnets (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. Liquid nitrogen 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 are 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. For a review of semen collection methods for various mammals see Watson (1978).

8.1. Sperm collection methods

8.1.1. Conventional semen collection methods

Collection of bull, ram, buck and stallion semen can be performed using an artificial vagina (AV), while boar semen will be collected using the hand-glove method. These can, if need be, performed on-farm if the animals have been trained; otherwise electro- ejaculation could be considered for the species mentioned, excluding boars and stallions. Also, the abdominal stroking technique can be used for semen collection in poultry. For the basic supplies and equipment needed for semen collection and freezing see Appendix A. For procedures for cryopreserving sperm from common livestock species see Appendix B.

8.1.1.1. Collection using an artificial vagina (bull, ram, buck, stallion)

Before collection, the AV should be prepared with volume of warm water (usually between 42° and 48°C inner wall temperature depending on animal body temperature) so that sufficient physical pressure will stimulate the glans penis of the male. The AV should remain as close as possible to this temperature throughout the semen collection process. The collection liner of the AV should then be lubricated with a nonspermicidal sterile gynaecological lubricant.

A teaser animal of the same species is recommended for the collection process. This animal is restrained and the male from which semen is being collected is allowed to mount one to two times for false collections. This procedure will allow the male to become aroused and increase the 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 is taken into the laboratory and the semen is processed for insemination or cryopreservation.

8.1.1.2. Gloved-hand technique (boar)

In this case, the boar is allowed to mount in the teaser animal or mounting dummy. The penis is grasped so that pressure is applied to the gland penis with the smallest finger of the collector's hand and the ridges of the penis are between the collector's fingers. 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.

8.1.1.3. Abdominal stroking technique (poultry)

In poultry, semen collection is performed by the abdominal massage method previously described by Burrows and Quinn (1935). Males are restrained and stroked from behind the wings towards the tail with firm rapid strokes. The male responds with the tumescence (erection) of the phallus and then the handler gently squeezes the cloaca expressing semen through the external papillae of the ducti deferentis. 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.

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

In general, the conventional methods described above are preferred for semen collection, as they tend 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 insulated using the hand of the collecting technician. It is recommended to follow the electro-ejaculation instructions provided by the manufacturer. Training and experience are needed to be proficient in semen collection using this methodology.

8.1.3. Epididymal sperm collection

Attempts have been made at harvesting epididymal sperm from the live intact farm animal males over the years by either cauterization or flushing the lumen of the cauda (tail) epididymidis with a hypodermic needle and a plastic syringe. Of these two approaches, the best success has been reported with catheterization of the cauda epididymidis in the mature male. In most males, the catherization 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 have been removed they are placed in a sterile resealable (zip-lock) plastic bag, then into an empty styrofoam cooler and transported to the laboratory for processing. For longer distance transport of bull testes, the temperature during transport is usually adjusted by 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 7°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 intact testes of some animals (e.g., dog) can be frozen in a plastic Zip-Lock bag shortly after collection and allow viable sperm to be collected after thawing the testes (Graff *et al.,* 2000).

8.2. On-farm processing and shipping samples to the processing center

Following collection, the samples can be immediately cryopreserved or transported to a laboratory (up to 24 hours holding time) for cryopreservation. The protocols for this are species-specific and thus, require specific medium as well. A shipping container should 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.

8.3. Collection and processing semen by species

8.3.1. Bull

8.3.1.1. Collection

The teaser female or steer must be restrained in a sturdy collection chute that affords protection to the collecting technicians prior to, during and following the bull collection procedure. False mounting is recommended for all bulls, regardless of age.

Electro-ejaculation can also be use to collect semen. The probes of electro-ejaculators differ in size according to species. 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.

8.3.1.2. Processing

Semen samples are diluted to the final desired sperm concentration in 37°C one-step medium (Bull: 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) or with the two step-procedure (Appendix B). The samples are placed in the shipping container, if necessary, and cooled to 5°C. Following cooling (within 2 to 12 hours) the samples are loaded into preprinted straws and frozen in LN_2 vapor (4.5 cm above the liquid for 12 minutes) and plunged into the liquid for storage. For a review of semen collection, processing and handling for bulls see the classic laboratory manual by Herman *et al.* (1994).

8.3.2. Ram and buck

8.3.2.1. Collection

As for bulls, the AV can be used for collection of ram and buck semen. Bucks and rams often quickly learn to mount a restrained ewe, and intromission and ejaculation are very rapid.

A second option is the collection of semen by electro-ejaculation. 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 electro-ejaculator is lubricated and 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. Usually the ram is not stimulated more than three times during the collection process and allowed at least 1 hour between collections. After ejaculation, the semen is covered to maintain its temperature and taken to the laboratory for processing.

As described in Section 8.1.3., the post mortem collection of epididymal semen is an third alternative to be used if a valuable male has died or there are no trained rams and bucks available the semen is assumed to be more valuable than the living male. Testes, collected after slaughter, can be transported to the laboratory in a thermos package at body temperature (38°C) or may be put in plastic bags and chilled. The caudae epididymides are separated from surrounding tissue, sliced and suspended in Salomon's one-step freezing medium at a 1 to 4 dilution rate. 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).

8.3.2.2. Processing

In the first step, dilute the samples in one-step cryopreservation diluents at 37°C (Ram: 300mM Tris, 28mM glucose, 95mM citric acid, 2% (v/v) glycerol, 15% egg yolk, 1 mg/ml of streptomycin sulfate and 0.06 mg/ml of benzylpenicillin). For the buck, the same medium is used for rams (except prepared with 2.5% egg yolk) and cooled with the same procedure as with bull semen. Following cooling to 5°C, the samples are loaded into preprinted straws and frozen in LN2 vapor (4.5 cm above the liquid for 7 minutes) and then plunged into LN2 for storage.

8.3.3. Stallion

8.3.3.1. Collection

Stallion semen samples can be collected by AV using a “phantom mare”. A phantom mare is a man-made object contructed to mimic the size and shape of a real horse. Different types (e.g., Colorado, French, Missouri and Roanoke) and sizes of AVs are available according to the stallion breed size. Protective head gear such as a helmet is needed for all persons handling the stallions prior to, during and following collection.

Stallions should not be electro-ejaculated due to the physical discomfort and poor semen quality derived from this method.

8.3.3.2. Processing

The semen samples can be diluted in 37°C holding medium. There are traditional standard (e.g., Kenny Extender: 49 g D-glucose and 24 g non-fat dry milk in 1 l of water) and now commercially media available. For example, for the domestic stallion the ejaculate can be diluted to 50 x 10^6 sperm per ml with 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) centrifuged (800 x g for 9 minutes) and the supernatant removed.

The resulting pellets are suspended in a small amount of SMED and the sperm concentration is then determined. The sample is then diluted 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) and cooled to 15°C in a shipping container, so that it can be held up to 24 hours. After holding, the sample is cooled to 5°C over 2 hours and diluted with SMED-glycerol, so that the final dilution is 4% glycerol (up to a 1:1 dilution [v/v]). The samples are loaded into printed straws and frozen in LN_2 vapor (4.5 cm above the liquid for 10 minutes) and then plunged into LN_2 for storage.

8.3.4. Boar

8.3.4.1. Collection

For an overview on methods of evaluating boar semen quality see Woelders (1991) or Colembrander *et al.* (2000). Once the boar has mounted the collection dummy, the penis is grasped so that pressure is applied to the glans penis with the smallest finger of the collector's hand and the ridges of the penis are between the collector's fingers. The penis needs to be fully extended prior to semen collection. After the initial fractions of semen are ejaculated, the sperm rich ejaculate should be collected into a 37°C insulated container covered with two layers of sterile gauze to remove the gel fraction.

8.3.4.2. Processing

For example, the ejaculate can be diluted in 37°C Beltsville Thawing Solution (BTS) (205 mM of glucose, 20.4 mM of sodium citrate, 14.9 mM of sodium bicarbonate, 3.4 mM of EDTA and 10 mM of potassium chloride), placed in the shipping container and cooled to 15°C. After cooling, the samples are centrifuged (800 x g for 10 minutes) the pellets are combined and the sperm concentration determined. Boar sperm samples can be diluted 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 10^6 sperm per ml and cooled to 5ºC over 2.5 hours. Samples are then diluted with BF5 freezing extender (91.5% of CE, 6% glycerol, 2.5% Equex Paste [v/v]) to 500 x 10^6 sperm per ml and loaded into 0.5 ml preprinted straws. The samples are frozen in LN2 vapor (4.5 cm above the liquid for 15 minutes) and plunged into LN2 for storage.

8.3.5 Poultry

8.3.5.1. Collection

Semen is collected by the abdominal massage technique (Burrows and Quinn, 1935). The male donor is carefully restrained by one person between his or her arms and body, while a second person collects the semen. This person strokes the abdomen a few times with his hand over the back towards the tail. The behaviour of the animal indicates its readiness to ejaculate and the semen collector will hold the pre-warmed tube underneath the cloaca, while lightly squeezing the cloaca with two or three fingers.

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.

8.3.5.2. Processing

The avian ejaculate will usually have a volume of approximately 0.5 ml and will usually contain from 2 to 6 x 10^9 spermatozoa per ml (depends on breed and the male). The ejaculate generally is

quite viscous. After collection, the volume is estimated and an equal volume of pre-warmed Lake's extender is added to wash down the semen and supply an initial pre-dilution. The semen should be discarded if it is watery or contaminated with blood or feces or if the volume is significantly less than 0.5 ml.

8.4. Embryo collection

8.4.1. Superovulation of donor females

Ideally, to maximize effective population size 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 Saumade *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 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 cattle donors today are presented in Appendix C. For successful embryo recovery, the current recommendations for number of artificial 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). Table D1 in Appendix D, presents data that gives 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.

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.

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1 http://www.fao.org/DOCREP/004/T0117E/T0117E00.htm

2 http://www.fao.org/DOCREP/004/T0120E/T0120E00.htm

Expected pregnancy rates from embryo transfer in farm animal species are presented in Appendix D, Table D2.

8.4.3. Stages of embryo development

Embryos continually develop through various morpholocial stages after *in vivo* fertilzation. 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[1]	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[2]

[1]Days from standing estrus.

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

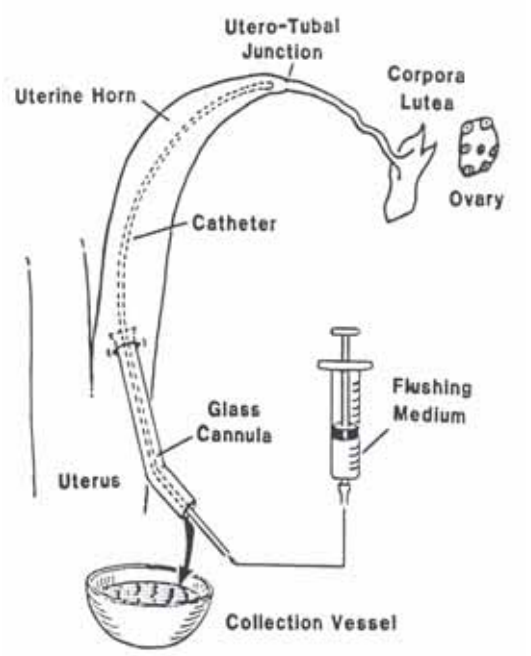
An embryo technician must be able to recognize not only the stage of embryo morphological development but also to assess embryo quality before selecting and transplanting embryos. This ability to make this judgment can be developed only with experience gained in assessing and grading embryos in the laboratory. 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).

8.4.3. Surgical embryo 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. 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 (Figure 8.1) (see review by Betteridge, 1977).

Today, surgical embryo collections for swine, sheep and goats are usually done at either at a commercial ET transplant units. Information on superovulation procedures and surgical procedures available are presented in the FAO document No. 115 for sheep and goats (available in French and Spanish only). 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. Commonly used approach to collect embryos (morulae and blastocysts) from sheep and goat donor females (drawing by R A. Godke).



8.4.4. 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 E. The body of the uterus and uterine horns simultaneously can be flushed using one flushing procedure, often called “Uterine Body Flushing” or “Body Flushing” or each uterine horn can be flushed separately using two flushing procedures and 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 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 required, 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 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.

8.4.5. 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, however, in other countries the use of this ET technology 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.

8.4.6. General recommendations on embryo collection

To maximize efficiencly, the collection, processing and storage of embryos must be carried out by a trained professional. In addition, many countries will have specific regulations on who can perform ET. Technicians will need to undergo special training relating to sanitation and techniques. The ET 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 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 E). 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 ET team. This entrusts a very high responsibility on a competent ET 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 ET 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 International Embryo Society's (IETS) recommendations for the sanitary handling of *in vivo*-produced embryos before beginning embryo collection (see Stringfellow, 1998). 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 of the donor animal, its herd or flock mates and the general environment in which the animals are kept for the presence of diseases. 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's Animal Production and Health Paper No. 23 on Disease Control in Semen and Embryos[3]

8.5. 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 cryoprotective agent, 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 F. 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 based upon a reversible dehydration of the cells that prevents the least damaging effect of intracellular ice crystallization. Most of the

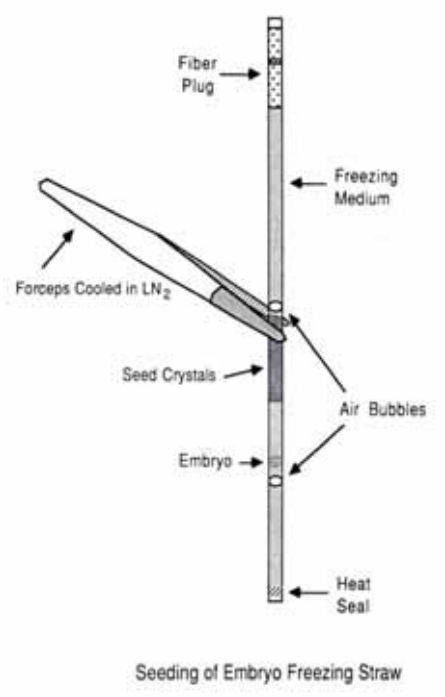
[3] http://www.fao.org/DOCREP/003/X6525E/X6525E00.HTM

technicians that use the slow freezing technique use it with 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). 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).

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.

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



8.6. 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 and Fahy, 1985; Rall, 1992). Vitrification is a more rapid procedure that uses a high concentration mixture of cryoprotective agents. 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 often 10 to 15% less than the slow freezing technique until one has 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 vitrification may be have some advantages over standard 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.

8.7. 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 demi-embryo of the pair using an H-Y antibody procedure. Then each of demi-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 demi-embryos and control demi-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 embryo biopsy 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 the seedstock producers and breeding studs to identify genotypic and/or phenotypic traits of the embryo by using quantitative trait loci (QTL) technology. The potential for use of QTLs to select the appropriate embryo to transfer would be of marked benefit to the commercial seedstock producers. Various companies have recently started making QTL-based technology available on a commercial basis to livestock producers. This technology will be particularly useful for cryobanking with the objective of gene introgression (See Section 6.3).

Today, research efforts are directed toward minimally invasive biopsy procedures to enable harvest of embryonic cells for use in genetic diagnosis and identifying positive genotypic traits by using QTL technology. The ability to perform genetic selection on embryos prior to transfer rather than performing phenotypic selection on animals after birth, puberty, or lactation (depending on the trait of interest) would clearly be of benefit to the seedstock and commercial livestock producers. The use of new genomic technology will likely enhance this selection ability (see Berardino, 2001). All indications are that the use of production-trait QTLs to select embryos for transfer to recipient females will be made commercially livestock producers in the near future. For cryoconservation programmes, these technologies may also be useful for selecting animals or embryos that will provide the most genetic variability to the population.

8.8. Oocyte collection

8.8.1. Conventional oocyte collection

Slaughterhouse ovaries are often used to harvest oocytes for research purposes and can be an option for cryoconservation, especially if the germplasm is expected to remain within the country and strict adherence to OIE sanitary standards for export is not necessary. The ovaries of cattle are collected immediately after slaughter, placed in recloseable plastic zip-lock bags and kept warm enroute to the laboratory facilities. Do 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 G), 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 H and I). This methodology can be used to collect oocytes from nearly all species of livestock. This approach to oocyte collection 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 all ovaries from 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.

8.8.2. Transvaginal ultrasound-guided oocyte collection (TUGA)

Oocytes may be collected from live donors via follicle aspiration using one of the 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 used more commonly in cattle, buffalo and horses. It is often referred to by its common name, “ovum pick-up (OVU).

TUGA was originally developed in humans to retrieve oocytes by using ultrasonography to evaluate the ovary and to transvaginally guide a needle into to each visible ovarian follicle (e.g. Wikland and Hamburger, 1984; Dellenback *et al.,* 1985). Fresh *in vivo* oocyte are then aspirated from the follicle and 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 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

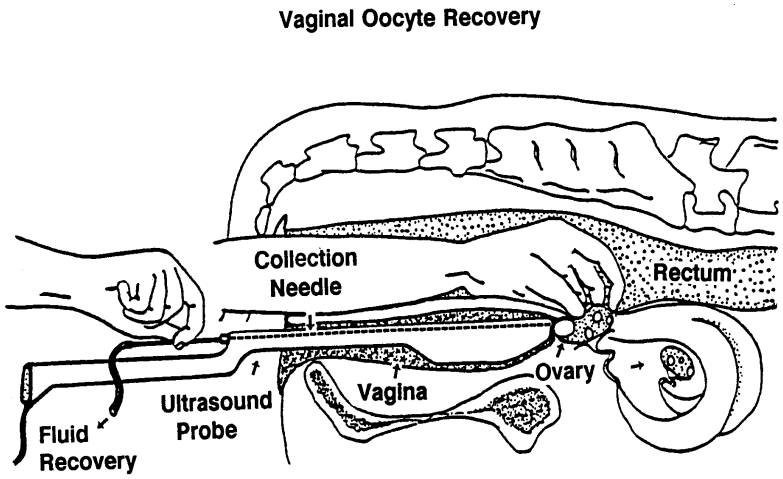
production. In addition, TUGA can be used on animals before they reach sexual maturity. Oocytes from prepubertal sheep and cattle have produced IVF offspring (see Looney *et al.,* 1995; Bols *et al.,* 1999). Oocytes from near-term bovine fetuses and new born calves are being harvested from farm animals 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.

8.8.2.1. 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 (by 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 phosphate-buffered saline (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). The number of oocytes collected per female can be increased by treating the female with gonadotropic hormones prior to the aspiration procedure in cattle and horses. *In vitro* production of embryos generally results in 1 to 3 bovine embryos for transfer per oocyte collection procedure for nonstimulated donors.

8.8.2.2. 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.

8.8.2.3. TUGA in the horse

The horse has presented a unique problem for researchers working in the assisted reproductive technology area. As noted prevoiuslsy, 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.

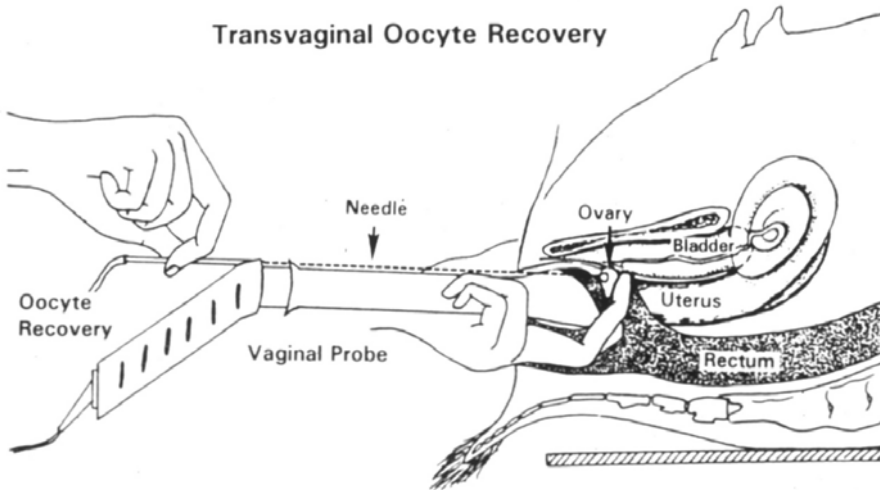
Even though only one follicle normally matures and ovulates during an estrous cycle, mares also are thought to have one or two waves of multiple follicles during that estrous cycle. Once again, this developing follicle population makes it possible to use TUGA to collect oocytes from live mares (see Brück *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. 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 appears to be the method of choice at this point to produce horse embryos in the laboratory.

8.8.2.4. TUGA in small ruminants

Goats are another farm animal species in which *in vitro* embryo production 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 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 noninvasive 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.

8.8.2.4. 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 (Locarelli *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. Currently, TUGA is being used to valuable oocytes from minor farm animal breeds, from domestic females representing rare bloodlines, clinically infertile females and reproductively senescent cows.

8.9. Harvesting animal cells and tissues for the gene bank

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 or to obtain genetic and health information about the animals sampled.

8.9.1. 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. Methods for collection of blood are presented for mammals and avian species in Appendix J, Section a.

Collection of two vials of whole blood (total of 12 to 14 ml) is recommended at the time of collection any other germplasm for animals selected for the gene bank. 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-labeled sterile vials for use in nuclear transfer. The vials are then frozen in nitrogen vapor and stored in LN2.

8.9.2. Somatic cells

Somatic cell tissue may also be sampled from live animals or from animals shortly after death for subsequent use in DNA analyses or cloning. One approach is to aseptically remove thin strips of skin from the body surface (e.g., shoulder area) of an animal with a sterile scalpel blade and place them in a pre-labeled 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. Animal root hair samples may be collected for DNA analysis in the short-term but are not suitable for long-term use. 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. For methods of harvesting skin samples from goat, sheep and cattle see Silvestre *et al.,* (2004). For procedures on harvesting and extracting DNA from somatic cells for genetic analyses see Appendix J Section b).

8.9.3. Gonadal tissue (poultry)

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).

8.10. Post-collection processing of tissue samples

After arriving at the laboratory, the tissue samples should be sliced into small pieces preferably 0.25 to 1.0 cubic mm 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.

The samples (4 to 5 pieces) are then washed in 20% fetal calf serum-PBS (FPBS) and placed in vitrification solution (VS; 20% [v:v] ethylene glycol, 20% [v:v] dimethylsufoxide in FPBS), loaded 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), sealed and plunged vertically into LN2.

Samples are thawed by holding the straw in LN2 vapor for 10 seconds and then submerged 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 or used for DNA extraction.

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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 is to minimize the risk of spreading diseases while collecting germplasm in the field 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 center 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 wavier 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 center 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 center 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 artificial vaginas 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 LN_2 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 traveling 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. Latex 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 recommened 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

Cross contamination of samples in LN_2 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 is 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.

If the sanitary level of stored samples is questionable, then the semen straws or other storage devices can be cleaned with ethanol and allowed to dry following thawing. Separate storage of samples of uncertain sanitary status should also be considered and specific LN_2 tanks designated for quarantine purposes should be used. 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 be segregated. Likewise, if samples collected in the field by the gene bank laboratory personnel are considered suspect for sanitation and health issues, then they too 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.

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 catalog, 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 because potential requestors have a way to view 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 genetic resources programme and makes it easier for the diverse users to access collection information and make use of the germplasm collection.

Databases for gene bank management can be diverse with a wide range of variation. 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 scheme will be developed which 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 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; and

- 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.
- Inter-operability - 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, which 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

1. 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 genebank.

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*				
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		

*Thawing instructions may be included as additional information.

11. REPRODUCTIVE METHODS FOR THE USE OF STORED GERMPLASM

Collection and cryopreservation of germplasm and depositing it in the gene bank are the necessary steps for creation of a gene bank, but this is only half of the process. The main purpose of creating a gene bank is to ensure the germplasm of interest is available for utilization in the future. Various technical steps are required to generate a live animal from the stored material. The difficulty of this process will depend upon a number of factors, including the type of material stored and the livestock species.

11.1. Artificial insemination

Representative procedures for thawing semen from various farm animals are presented in Appendix B. It should be noted that thawing protocols within species vary from laboratory to laboratory and therefore the protocols should serve as a general guideline and be evaluated, fine-tuned and optimized under local conditions prior to starting an in-field AI project.

For a historical review of the events and milestones leading to successful AI programmes for farm animals as we know them today, see the work of Foote (1999). Since the first calves resulting from frozen-thawed sperm were reported in England (see Stewart, 1951; Polge and Rowson, 1952), AI has become one of the important tools for genetic improvement in the world.

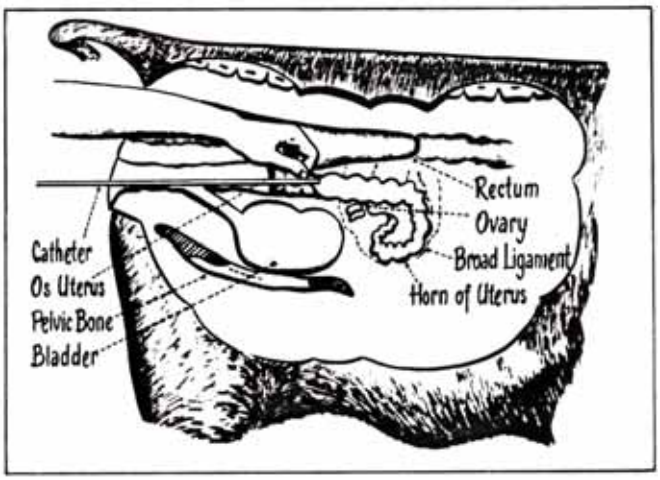
11.1.1. *Conventional* AI

Due to obvious differences in size, structure and reproductive physiology, approaches for administration of AI differ among species. These next sections highlight some of these differences.

11.1.1.1. Cattle and Buffalo

The learning curve for developing successful artificial insemination skills is the shortest for horses, cattle and swine. Hands-on short courses can teach the beginner the basics (Figure 11.1) and it then only takes serious practice thereafter to become proficient. For a review of the basic equipment, a procedure for detecting estrus and the basic steps of AI procedures for cattle see Appendix K. Sections a, b and c.

Figure 11.1. With the glove hand placed in the cow's rectum the Al pipette is inserted through the lumen of the cervix to deposit tie semen into the uterus.



Proficient AI, especially in sheep, goats and cattle, requires a great deal of hands-on practice. A simple radiographic method to verify semen deposition in the uterus during cattle AI training has been found to be very helpful for the beginning technician (Peters and Senger, 1983).

11.1.1.2. Horses

The AI procedure for the horse is very similar to that of that of cattle, except the volume of extended semen and the insemination pipettes are larger. AI in the mare is generally considered to be easier to perform because an estrual female has a relaxed cervix that is easier to penetrate with the insemination pipette for semen deposition. The volume of extender inseminated ranges from 40 up to 100 ml per insemination dose. The mare is usually inseminated the second day of standing estrus and every other day thereafter until she is no longer in standing estrus. Mares that are inseminated with cooled or fresh semen have a greater fertility rate and larger number of embryos recovered than do mares mated with frozen-thawed semen (Squires *et al.,* 2003).

11.1.1.3. Small ruminants

The learning curve is somewhat longer for becoming successful at AI in the goat, but most technicians agree the most difficult farm animal to successfully inseminate artificially is the domestic ewe. In the goat and sheep, the body frame and reproductive tract size are the primary factors influencing the ease or difficulty in completing the insemination process. In the ewe, for example, the anatomy of the cervix (annular rings) and the pathway through the cervical canal are given as the reasons for the difficulty encountered with the insemination of both mature young and even older ewes.

For a review of the basic AI procedure for small ruminants see Appendix L.

11.1.1.4. Swine

Artificial insemination in swine is considered relatively easy when compared to sheep and goats. The thawed semen is extended to 50, 75 or even 100 ml with extender and placed in appropriate-size, clean, plastic “squeeze” bottles (available commericially) and maintained until the time of insemination at room temperature. At the time of insemination, the squeeze bottle is attached to a swine flexible breeding spirrette (available commercially) and the lubricated spirrette is placed into the vulva, through the vagina and locked tightly into the cervical canal of the female. The semen is then slowly deposited into the uterus by placing moderate manual pressure on the squeeze bottle until all the semen is removed from the bottle. Slowly remove the spirrette and allow the female to return to her paddock or pasture at her own pace to reduce semen back flow. When done correctly, the female will be in standing estrous at the time of AI, she usually does not offer much resistance during this procedure.

With twice a day estrus detection (morning and evening), sows are usually inseminated 24 hours after the onset of standing estrous and then every 12 hours thereafter until the female is no longer in standing estrus. Gilts are usually inseminated 12 hours after the onset of standing estrus and then every 12 hours thereafter until the female is no longer in standing estrus. It should be noted that the fertility obtained with frozen semen is less in sows and gilts and the litter size is approximately 2-3 piglets less than with fresh semen (Martinez *et al.,* 2005).

11.1.1.5. Chickens and turkeys

After thawing, proceed to insemination of hens immediately with a total dose of 600 x 10^6 sperm (2 straws) per insemination, with 2 inseminations per hen per week. The insemination volume should be 60 to100 µl (if larger volumes of sperm are delivered per insemination then sperm will be partly expelled). For basic procedures on artificial insemination in chickens and turkeys see Etches (1996).

11.1.2. Conventional AI with epididymal sperm

Artificial insemination with fresh or frozen-thaw epididymal sperm has been reported to produce live offspring from the horse (Barker and Gandier; 1957; Papa *et al.,* 2008), goat (Blash *et al.,* 2000), sheep and cattle (Guerrero *et al.,* 2009), as well as several exotic species. No significant differences in pregnancy rates with frozen ejaculated and frozen-thawed epididymal sperm in

rams and bulls have been observed. Developing this methodology should not be overlooked for its potential in saving valuable germplasm.

11.1.3. Surgical insemination

When one has a choice, nonsurgical AI is the preferred approach to breed farm animals. The surgical approach (laparotomy and laparoscopic methods) has been reported to be successful in sheep and goats and has become the method of choice for larger production producers in some countries (e.g., Australia). Although surgical approaches to AI have been devloped for horses, cattle and swine, they are seldom used and usually only in problem breeding females.

Surgical AI has been successfully used to inseminate both goat ad sheep embryo donors over the years. Pregnancy rates are usually greater than with conventional AI. The number of fertilized embryos per donor female also tends to be increased by using this method, but the downside is that two surgeries are required on each female, for AI and to harvest the embryos. Not only is this approach more time-consuming and labor intensive, there is an increased chance of post-surgical adhesions occurring, which may lead to post-collection infertility of the donor animals. For expected pregnancy rates expected from both AI and surgical inseminations of farm animals see Appendix M.

11.1.4. Estrus detection for AI

One primary reason why AI fails at the farm level is simply poor estrous (heat) detection. Because it is not practical to continuously monitor females for signs of estrus, producers often use various aids for detecting standing estrus for insemination. Cryoconserved semen is likely to be particularly valuable, so ensuring its efficient use through proper estrous detection is very important. For cattle, pressure-sensitive mounting devices are commercially available, which are placed on the tailhead of females to detect the weight of one female mounting another and active an electronic transponder, change color or leave another easily visible indication of mounting. Potential drawbacks of these devices include loss from tailhead and occasional false positives (due to rubbing on trees, fences, etc).

Estrus detection in sheep and goats is done with a mature vasectomized teaser male twice daily (morning and evening). A teaser stallion is needed for effective detection of estrus in breeding mares. A vasectomized teaser boar is recommended (Godke *et al.,* 1979) for detecting estrus twice daily in sows and gilts. An experienced animal management staff is needed to handle the male teaser animals prior to, during and after detecting estrous, especially with boars and stallions.

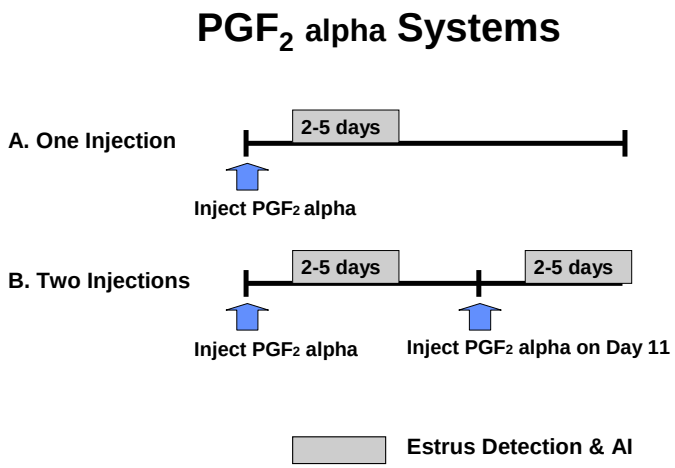
A second type of detection aid is animal marking devices. Physical marking devices include tail chalk or paint (often used on dairy cows), chinball marking harness (used on teaser or breeding bulls) (see Godke *et al.,* 1983) and placing paint (a bright color for each male) on the brisket of teaser/breeding males (often used on breeding rams).

11.1.5. Estrous synchronization methods

To save time and to increase efficiency of AI, estrous synchronization methods may be considered. Estrous synchronization schemes are most often used in cattle, sheep and goats. Generally, the easiest and often the cheapest method for synchronizing cattle in small groups for AI and embryo transplantation is with prostaglandin F_2 alpha (PGF_2 alpha) (Figure 11.1).

For some other popular estrous synchronization methods with various hormone agents used for cattle today see Appendix N. For a review of the estrous synchronization procedures in cattle (see Lamb *et al.,* 2001; Day, 2005).

Figure 11.1. For randomly cycling cattle with a corpus luteum, the simplest approach to synchronizing small groups of females for AI, use one dose of PGF_2 alpha (A) or two doses of PGF_2 alpha (B).



Estrous synchronization procedures are also often used to synchronize the estrous cycle of the recipient females with that of the donor females for ET. The day of the estrous cycle and the developmental age of the donor's embryos need to be on or close to the same day of the estrous cycle of the recipient females (e.g., day 7 of the cycle in cattle) to optimize transfer pregnancy rates. This factor must be considered when implanting embryos that had been cryoconserved in a gene bank, given the expected value of the genetic material.

For estrous synchronization methods for AI often used in sheep and goats see Appendix L. The post-weaning estrus (3 to 7 days after weaning of the piglets) is most often used to synchronize sows for AI. For cycling gilts, most often Pregnant Mare Serum Gonadotrophin (PMSG, known commercially as PG 600) is administered to females pre-treated with altrenoges (commercially known as Regu-mate) (see Stevenson and Davis, 1982; Estienne *et al.,* 2001). Mares are usually prepared for AI in small groups using a single injection of prostaglandin F_2 alpha administered during the luteal phase of the estrous cycle.

11.2. Embryo transfer

11.2.1. Thawing of cryopreserved 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. Although live offspring have been reported born from frozen-thawed embryos livestock of most common livestock species, the best success from this cryotechnology has been with cattle embryos, followed by sheep and goat embryos. Of all the major livestock species, it took the longest and the most intense research effort to produce live offspring from post-thaw embryos in pigs (Hayashi *et al.,* 1989, and others) and still today swine embryos remain the most difficult to freeze and thaw for offspring production.

Cryopreserved bovine embryos are most often thawed rapidly by placement into a water bath for 8 to 30 seconds (the time depending on the freezing procedure), and then the cryoprotective agent is removed before transfer of the embryo into a synchronized recipient female (see Appendix F). Pregnancy rates following transfer of excellent quality frozen-thawed embryos are generally only slightly less than obtained after transfer of fresh bovine embryos. The success rate of obtaining a

live offspring from the transfer of good quality of frozen-thawed embryos ranges between 50 and 70%.

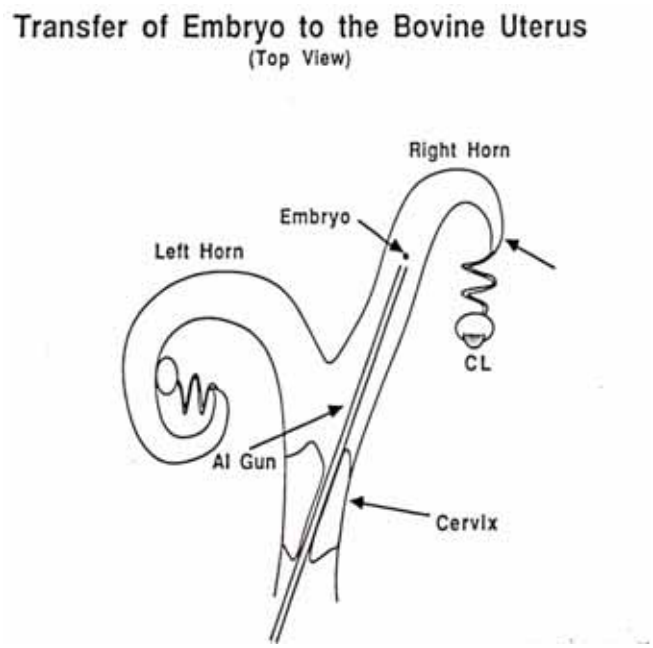
One of the more recent developments in embryo freezing in the cattle industry is the direct transfer method (Leibo, 1983). The primary advantage of the direct transfer method is that the embryo is transferred to a recipient female immediately after thawing without the need to first remove the cryoprotective agent from the embryo (Leibo, 1984; Voelkel and Hu, 1992).

11.2.2. Nonsurgical embryo transfer in cattle

Cattle embryos (early to late blastocysts) are most often transferred on day 7 or early day 8 of the estrous cycle (onset of standing estrus = day 0 of the cycle). The recipient female should have an identifiable corpus luteum (CL) at the time of ET. The recipient should be calmly restrained in a squeeze chute and a tail-block epidural administered (5 to 8 ml of lidocaine) ~10 minutes before transferring the embryo. If a good quality CL is present, the vulva should be washed with room temperature water and dried clean paper towels.

The Cassou gun (apparatus) (see Cassou, 1964).containing the embryo in a 0.25 ml plastic straw is placed through the vagina to the external *cervical os*. The protective sheath on the Cassou gun is then pulled back and the gun manipulated through the cervix into the body of the uterus using manual manipulation via the rectum. It is important that the embryo be carefully deposited into the uterine lumen of the uterine just past the bifurcation ipsilateral to the CL, being careful not to irritate the uterine endometrium with the Cassou gun (Figure 11.2). It is important to place the cattle embryo into the uterine horn ipsilateral to the CL to maximize the chances for a transplant pregnancy.

Figure 11.2. The cattle embryo is placed into the uterine horn on the same side of the uterus with the ovary having the corpus luteum (CL) (Drawing from R. A. Godke).



11.2.3. Embryo transplantation in other farm animal species

Embryo transplantation in the horse is similar to that of the cow, except that the transfer pipette is generally larger in diameter because of the large-size horse embryo at this stage, and the embryo is placed in the body of the uterus (see Squires *et al.,* 2003) Unfortunately, frozen-thawed later stage equine embryos often do not produce a viable pregnancy and are thus rarely used under field conditions. For an overview on the status on equine embryo transfer see Squires *et al.,* 1999, 2003.

Sheep, goat and swine embryos are generally transferred via a mid-ventral laparotomy approach, so highly-trained professionals and proper surgical facilities are needed for these transfers. Sheep and goat later-stage embryos (blastocysts) are carefully placed into the lumen of the distal end of the uterine horn near the uterotubal-junction (UTJ) ipsilateral to the CL or into both uterine horns if the female has multiple CL (one or more CL on each ovary). Later-stage (balstocysts) swine embryos are all placed into the lumen of ether of the two uterine horns, since swine have embryo transuterine migration before starting the implantation process.

For a manual on pig embryo transfer procedures see Fuku (1998). Also, note recent advances in swine embryo transfer see Youngs (2001); Hazeleger and Kemp (2001).

11.3. Other procedures with current oppotunities for use 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;). Some of the following are options that can be considered already today when performing cryobanking, although they generally require a great deal of expertise or will need to rely on future development to increase efficiency and decrease costs:

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

Initial *in vitro* embryo production (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. The IVF procedure involves harvesting the oocytes from the donor's ovaries and fertilizing them *in vitro* (Appendix J). 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% (Zhang *et al.,* 1992, and others). 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 efficient IVF procedures 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 cost of production.

IVF in the mare has not developed to the level for in-field use as expected. Although several offspring have been 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 apparently longer than 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. Now that repeatable oocyte retrieval methods are being fine-tuned, it is likely these procedures will become routinely used to obtain oocytes for further gamete and embryo research and also by seedstock producers for *in vitro* embryo production from farm animals in the commercial sector.

11.3.2. Cloning via nuclear transfer

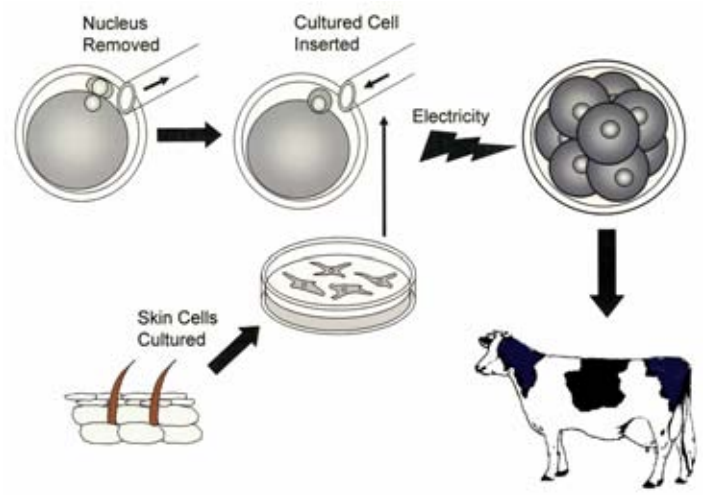
It has long been a dream of scientists to be able to produce genetically identical animals from differentiated mammalian cells obtained from genetically superior animals. The first nuclear transfer offspring born in the world from a mammal (sheep) was reported by Willadsen (1986) from Cambridge, England. Although these nuclear transfer lambs were derived from early stage, undifferentiated embryonic cells (<7 days of age), the ability to produce live healthy nuclear transfer offspring was lauded as a breakthrough by the scientific community. During the next 10 years, researchers produced live nuclear transfer offspring from all the farm animals (except the horse), using methodologies similar to Willadsen (1986).

Subsequently, other lambs were produced using more advanced differentiated embryonic cells as donor cells (Campbell *et al.,* 1996). This breakthrough ignited a flurry of research activity surrounding the ability to use differentiated embryonic and adult somatic cells as donor cells for nuclear transfer procedures. The first offspring (i.e. “Dolly the sheep”) derived from an adult somatic cell nuclear transfer SCNT) was reported in February of 1997 (Wilmut *et al.,* 1997).

Other researchers in the world continued this efforts and independently produced SCNT offspring from a variety of livestock and other species. Today more than 16 animal species have been cloned in the world. For the basic procedure for somatic cell cloning in cattle see Figure 11.4.

Until recently, SCNT was characterized by extremely high cost, low success rates, problems with pre-term and neonatal offspring viability and the existence of physiological problems such as the “large calf” syndrome in cattle. Therefore, SCNT was initially not adapted by the commercial sector. Some aspects of these problems have been addressed in several species, however, and some progress has been made. For example, clones have been produced from cells of previously cloned cattle. A Japanese research groups has reported an 80% success rate from transferring SCNT cattle embryos (Kato *et al.,* 1998). SCNT has been recently used in an attempt to save an endangered cattle bloodline (Wells *et al.,* 1998), endangered hoofstock species (Lanza *et al.,* 2000; Loi *et al.,* 2001; Janssen *et al.,* 2004; Li *et al.,* 2006) and the exotic African Wildcat (Gomez *et al.,* 2004).

Figure 11.4. The basic procedure for somatic cell cloning (figure prepared by Dr. Allison Landry).



Cloning through somatic cell NT (SCNT) has developed to the point where it is available commercially for livestock. There several companies offering this service in different locations around the world (Table 11.1). SCNT technology is clearly here to stay, and it will likely impact breeding and reproductive management strategies in the in livestock industry in the years to come. It should be noted, however, that non-genetic factors influence the phenotype, so that all clones from same donor or cell line do not the same growth patterns and other traits during prepubertal growth and adulthood (e.g., Landry *et al.,* 2005; Behboodi *et al.,* 2005, others).

Table 11.1 Some examples of commercial animal cloning companies.[1]

Company	Nation	Website
Viagen	USA	www.viagen.com
Cyagra/in vitro Brazil/Goyaike	Brazil, Argentina	www.cyagra.com.br
TransOva Genetics	USA	www.transova.com
Cryozootech	France	www.cryozootech.com
Avantea	Italy	www.avantea.it

[1] This list of companies is presented for informational purposes only and should not be regarded as an endorsement by FAO with regard to the quality of the services provided by these companies. In addition, commercial animal cloning is a young and dynamic industry, so the companies involved, their names and contact information are subject to change over time.

For many livestock species it is now possible to conserve a population simply by taking samples of somatic cells (e.g., skin cells or possibly follicle granulosa cells) and cryoconserving them (or cell lines derived from them) for SCNT. Costs are currently prohibitive for most species and many countries, but this possibility is important for a number of reasons. First, the protocols for collecting samples of somatic cells are less demanding than for collecting semen and/or embryos. Second, the costly aspect of cryoconservation with somatic cells is production of live offspring, which may not be necessary until the distant future, when technological advancements will possibly increase efficiency and decrease costs. At present, however, cloning on an in-house scale large enough to regenerate live populations is limited to a small proportion of FAO member countries.

11.3.3. 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 (Uehara and

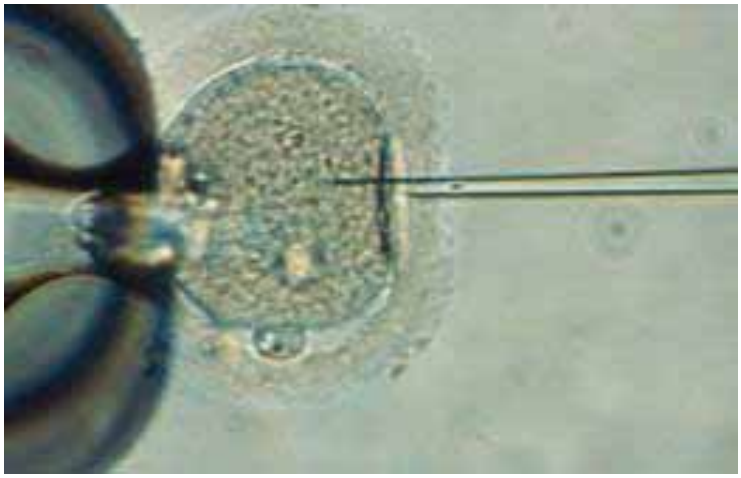
Yanagimachi, 1976; Markert, 1983, and others). Using this technique, the premise was that the ovum of the female would be activated by a microinjected sperm. The first ICSI experiments in mammals were conducted in rodent species, however, results were variable.

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 increase flexibililty. 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 LN_2 and greatly facilitate *ex situ* conservation of AnGR.

Figure 11.3. Intracytoplasmic sperm injection (ICSI) procedure (from R.A. Godke).



11.3.4. Oocytes

First results in mice and rabbit oocytes indicate that it might be possible in the future to generate offspring from frozen oocytes with *in vitro* techniques. The reported efficiency rates are still very low, with less than 10% of the frozen oocytes developing after fertilization and *in vitro* culture to produce viable offspring. Recent progress has been made using vitrification to successfully cryopreserve human oocytes for IVF (see review by Porcu and Venturoli, 2006). So far in domestic animals, only rabbits, calves and more recently foals have been born (Maclellen *et al.,* 2002) from cryopreserved oocytes. At this stage, frozen oocytes thawed for use in IVF embryo production has not been successful enough at this stage to be used under field conditions in the farm animals.

Frozen-thawed oocytes (in this case vitrified warmed oocytes) used with ICSI (in place of *in vitro* fertilization) have recently shown promise for use in clinical equine veterinary medicine. Oocyte

transfer to the oviducts of mares along with natural or AI has been successful producing pregnancies at both research and commercial equine fertility units (see Carnevale *et al.,* 2001, 2003)

In situations where the survival of a breed depends on only a few females, freezing oocytes could be attempted from those animals, especially those that yield no or poor quality embryos at collection. It may be possible to then use these frozen-thawed oocytes in the future (see Ledda *et al.,* 2001), once the technology is available, in an IVF program, or with ICSI directly into the ooplasm of the oocyte. This approach has now been reported to be successful using mouse oocytes (Kimura and Yanagimachi, 1995).

11.3.5. Ovarian tissue transplantation in poultry

The prospect of using cryopreserved ovarian tissue was previously discussed in Chapter 4. Recently, newly hatched chick ovarian tissues have been frozen, thawed and transplanted back to young females with the subsequent production of live offspring (Song and Silverside, 2006, 2007a, 2008a).

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). In the future, with ovarian tissue transplantation technologies, possibly another breed of chicken, when mature, could then produce eggs fertilized by the by sperm of the original breed and produce the original breed offspring (see Song and Silversides, 2008a,b; Liu *et al.,* 2009). Thus, with frozen male (sperm) and female gametes (stored frozen in intraovarian tissue) the complete genome could be recovered from one breed by another breed. Additional research this technology would offer new possibilities for future animal production systems but requires additional research and development, especially for mammalian species.

.11.4. Future prospects for cryoconservation

11.4.1. 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, thawed 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. These cells could thus be potential vectors for the transmission of genetic characters. However, even with an intensive research effort in recent years, there is at present no convincing evidence of the existence of true ESC from 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.

11.4.2. 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 they could be a means to saving genes from the male animals for cryoconservation of AnGR.

11.4.3. 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 migrate to the gondal ridge via the blood stream usually between 4 and 6 of incubation. During this migration interval the primordial germ cells can be harvest from the blood of chick embryos, 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 (Ono *et al.,* 1996 and others). Also, germline chimeras have 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.

11.4.4. 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 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 resulting in live offspring, each exhibiting distinct coat color patterns of both breeds (phenotype) and they each had a chimeric genotype (parthenogentic and IVF). 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.

11.4.5. 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.

11.4.6. *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 (Murakami *et al.,* 2006, and others).

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12. 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 U.S. 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.

12.1. 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.

12.2. 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 publically 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.

12.3. 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.

12.3.1. 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 publically accessible database. The submission of such information can take place after the information has been published.

12.3.2. Meeting the Request

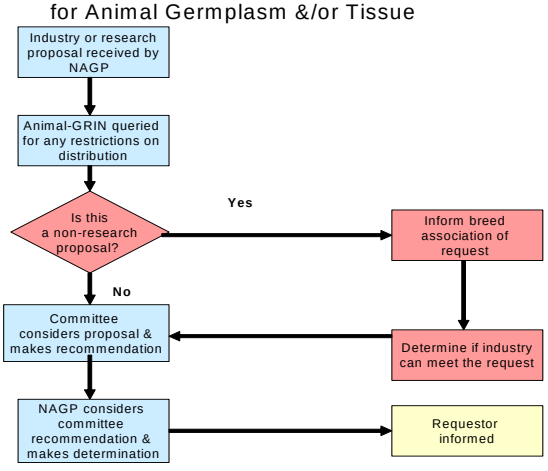
Once a request for germplasm or tissue is received and evaluated for its merit, the gene bank must also determine if sufficient quantities of the material exist in the gene bank to fulfill the request without compromising the Core Collection (see Chapter 3). If the requested cryobank material is also 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 1 details the process of reviewing non-research or research requests as used by the US (Blackburn, 2009).

Figure 12.1. Process used by the National Animal Germplams Program (NAGP) in the USA for reviewing industry or research requests for animal germplasm and/or tissue.

Figure 2. Process for Reviewing Industry or Research Requests for Animal Germplasm &/or Tissue



12.4. 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).

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13. 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); exchange of information relevant to the conservation, management and use of biological diversity; including information on research, training, surveys, and specialized knowledge (Article 17); and technical and scientific cooperation through, where necessary, appropriate international institutions, with special attention to capacity building (Article 18).

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. 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. It will also be necessary to organize training courses 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.

13.1. Topics to be taught in higher education

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 effective population size) 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 stored 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.

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.

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APPENDIX A. 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 scale (6 kg ± 1 g)
- Warm water bath (electric)
- Sperm counting equipment (hemacytometer 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 B. PROCEDURES FOR CRYOPRESERVATION AND THAWING OF SEMEN FROM COMMON ANIMAL LIVESTOCK SPECIES

Cryoconservation of Bull Semen

Freezing

1. Collect sperm (5 to 15 x 10^9 sperm per ejaculate). Avoid temperature changes in semen after collection.
2. Evaluate sperm concentration and determine final volume V for a concentration of 200 x 10^6 sperm/ml.
3. 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.
4. Cool to +5°C within 1 hour.
5. Add Diluent "2" up to final volume (Diluent B consists of Diluent A +11% glycerol).
6. Keep at +5°C for 2 hours.
7. Fill pre-printed straws of 0.25 ml with semen (25 to 30 million sperm/straw).
8. Transfer straws to LN2 vapour at -70°C/-100°C for 9 minutes.
9. Transfer straws to LN2 tank and store.

Thawing

1. Thaw a sample 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. Avoid temperature changes in semen after collection.
2. Evaluate sperm concentration and determine final volume V for a concentration of 100 x 10^6 sperm/ml.
3. 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.
4. Cool to +4°C within 1.5 hours.
5. Add Diluent "2" up to final volume (Diluent B consists of Diluent A + 11% glycerol). The final concentration of glycerol is thus 7%.
6. Keep at +4°C for 4 hours.
7. Meanwhile, fill pre-printed straws of 0.50 ml with semen (about 50-60 million sperm/straw).
8. Cool from +4°C to -140°C in 5 minutes, then plunge in LN2.
9. Transfer straws to LN2 storage.

Thawing

1. Thaw a sample to check for quality.
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. Collect sperm (4 x 10^9 sperm per ejaculate); select only those ejaculates with a mass motility >4.5 on a scale of 5.
2. Evaluate sperm concentration and determine final volume V for a concentration of 400 x 10^6 sperm/ml.
3. Add Diluent A (25. 75 g of lactose in 250 ml bi-distilled water +20% egg yolk) at 30°C up to $3/5^{th}$ of final volume
4. Cool progressively to +4°C over 2 hours (0.2°C/min)
5. 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-$5.5H_2O$ into 70 ml H_2O); then mix 9 volumes of the resulting solution with 1 volume of glycerol.
6. Add Diluent B in 3 equals parts, over 30 minutes, at 4°C up to the final volume
7. Keep the semen for 90 minutes at +4°C.
8. 0.25 ml plastic straws with semen.
9. Place straws horizontally in LN2 vapour at -75°C for 8 minutes.
10. 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 laparoscopic intrauterine insemination of pre-synchronized recipient.

Cryoconservation of Buck Semen

Freezing

1. Collect sperm (4 x 109 sperm per ejaculate in season); select only those ejaculates with a mass motility >4.5; keep at 32°C.
2. 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.
3. Discard the supernatant, and evaluate semen (wave motion, concentration). Calculate final volume (V). Repeat centrifugation under same conditions at 20°C.

4. Prepare Diluent A: 80 ml of a sodium citrate solution (194 mg glucose +3.52g sodium citrate +1.05g streptomycine +50 000 IU penicillin in 100 ml distilled water) supplemented with 20 ml egg-yolk.
5. Add V/2 of Diluent A to the pelleted sperm at 20°C.
6. Cool to +4°C within 30 minutes (at 0.5°C/min).
7. Add V/2 Diluent B (Diluent "1" + 14% v/v glycerol) in three successive steps with 10 min intervals.
8. Fill 0.25 ml plastic straws with semen.
9. Freeze straws in LN2 vapour for 5 minutes.
10. 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 estrous synchronized does.

Cryoconservation of Boar Semen

Freezing

1. Collect sperm (80 x 109 sperm per ejaculate). Discard the first emission of sperm; keep only the second one (about 200 ml, rich in spz with 40 x 109 total).
2. Filter the sperm through gauze to eliminate the bulbourethral secretions.
3. Dilute one volume of sperm with one volume of Diluent A (anhydrous dextrose, 37g; tri-sodium-citrate-$2H_2O$, 6 g; sodium bicarbonate, 1.25 g; EDTA diNa, 1.25 g; KCl, 0.75 g in 1 litre of bi-distilled water)
4. Cool to 15°C within 2 hours.
5. Centrifuge at 800 g for 20 minutes at 15°C. Remove supernatant, which is diluted seminal plasma.
6. Resuspend the pellet of sperm with about 10 volumes of Diluent B (fructose, 8.5 g; of sodium bicarbonate, 0.15 g; of cystein, 0.015 g; bi-distilled water, 116 ml; egg yolk, 34 ml; equex STM [Nova Chemicals], 1.69 g) to obtain a concentration of 3×10^9 sperm per ml.
7. Cool the suspension to 5°C over 2 hours.
8. 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.
9. Keep at 5°C for about 90 minutes during which time the semen is put into 0.5 ml. straws.
10. Place straws horizontally at 5 cm above the level of boiling LN2 for 15 miutes (this will ensure a freezing rate of about 20°C/minute down to -145°C.
11. 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 109 sperm per AI).

Cryoconservation of Stallion semen

Freezing

1. Collect sperm (~8 x 10^9 sperm per ejaculate) and filter on gauze.
2. Evaluate sperm volume and concentration.
3. Prepare Diluent A (1 volume of non-fat milk UHT +1 volume of a solution containing: 50 g/l of glucose, 3 g/l of lactose; 3 g/l of raffinose; 0.6 g/l of sodium citrate; 0.82 g/l of potassium citrate; 100,000iu/l of penicillin; 0.100 iu/l of gentamycine).
4. Add to Diluent A 2% egg yolk and mix three volumes of this solution with one volume of sperm at 32°C.
5. Cool to +4°C over about 1 hour (0.4°C/minute).
6. Centrifuge at 600g during 10 min at 4°C. Remove supernatant.
7. Prepare Diluent A (Diluent B supplemented with 2% egg yolk and 2% glycerol).
8. Re-suspend the pellet of sperm at 4°C with Diluent B to reach a final concentration of 100 x 10^6 spz/ml (50 x 10^6 sperm/0.5 ml straw).
9. Keep at 4°C for 30 to 45 minutes.
10. Fill 0.5 ml straws with semen.
11. Deep freeze straws in a programmable freezer: from +4°C to -140°C at a rate of 60°C/minutes. Alternatively, freeze straws horizontally in LN_2 vapor by keeping them at 4 cm above the level of the LN2 for 4 minutes
12. Plunge into LN2 and store.

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 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.
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 vapor 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 poultry semen

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.

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

	M.W.	g/l	mmol/l
Natrium-L-glutamaat.H_2O	187.13	19.2	102.6
Magnesium acetaat.$4H_2O$	214.46	0.7	3.3
Fructose	180.16	8	44.4
Kalium acetaat	98.2	5	50.9
Polyvinylpyrrolidone	40 000	3	0.08
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 or 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.

Freezing

Then, the tube is closed with a stopper to prevent evaporation (dehydration) and is placed in a thermostat-controlled cool box at 5°C. When all cocks are collected, the semen is transported to the laboratory for further processing and freezing. All further handling is performed at 5°C (cold room or open top cooler cabinet).

The sperm concentration of the pre-diluted semen is determined with a spectrophotometer or haemocytometer.

The semen can then be further diluted with Lake's diluent to a chosen sperm concentration (e.g., 1.8 x 10^9 sperm/ml).

Straws are printed. Then, half a volume of Lake-DMA is added to one volume of semen. Straws are filled and the straws are frozen.

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

Thawing

Straws are taken from LN2 and placed in a 5°C water bath. The straws must be moved vigorously through the water during 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 semen can be used directly for insemination after thawing. No need to first wash the semen free of the cryoprotectant

Freezing

1. Collect sperm (1.5 x 109 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.

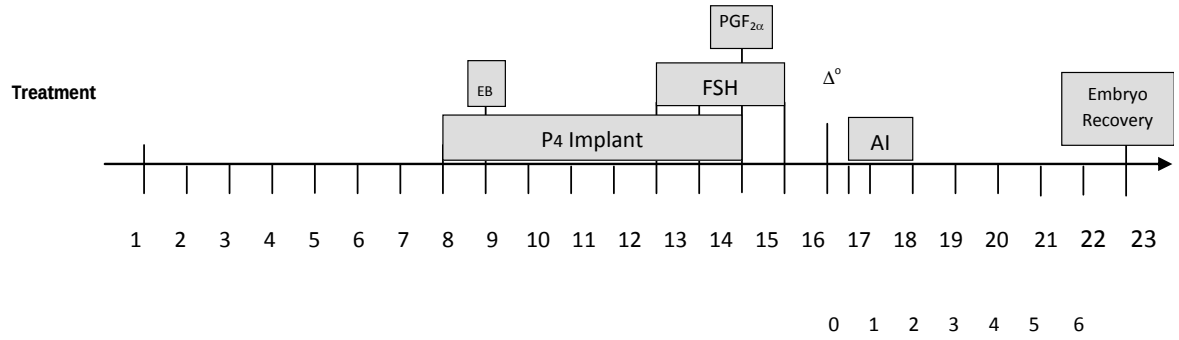
Thawing

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. 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.
3. Remove glycerol by centrifugation at 700 g at +5°C for 15 minutes
4. 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.
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 C. COMMONLY USED SUPEROVULATION SCHEMES FOR DONOR CATTLE EMBRYO RECOVERY

Scheme I



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

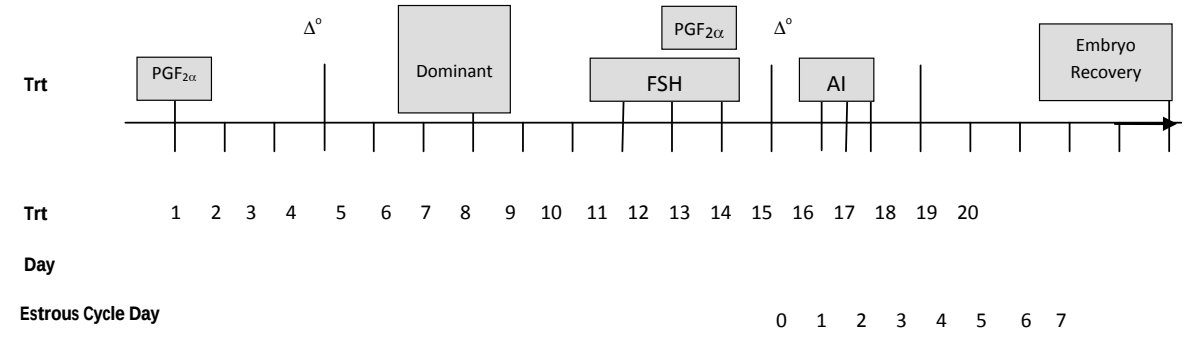
E = 2 to 5 mg of estradiol benzoate injected. This agent may be illegal to use in some countries.

Δ^o = Standing estrus.

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

FSH = Follicle Stimulating Hormone

Scheme II



APPENDIX D. NONSURGICAL AND SURGICAL EMBRYO COLLECTION AND TRANSPLANTATION IN FARM ANIMALS OF FRESH AND FROZEN-THAWED EMBRYOS

Table D1. Nonsurgical versus surgical embryo collections[a]

Embryo Collection Type	Cow	Sheep	Goat	Sow	Mare[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 use	yes	No	no	no	yes
Surgical					
Difficulty of the procedure	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 use of surgical collection	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 technicials 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 D2. Nonsurgical versus Surgical Embryo Transplantation of Embryos 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
Recommendation 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
Recommendation 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 technicials 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.

APPENDIX E. 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 F. 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 swine embryos is more difficult, although there have been some recent advances in this area. Freezing early-stage horse embryos has been reported but freezing later-stage equine embryos has still has not been mastered for in-field use at this time.

1. Cryopreservation of Bovine Embryos

a. Freezing Procedure.

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. Wash the embryos from one donor in 10 consecutive baths of phosphate-buffered saline (PBS). Equilibrate the embryos at room temperature for 10 minutes in phosphate-buffered solution with 10% fetal calf serum (FCS) and 10% glycerol.

3. Place the embryo between two air bubbles in a 0.25 ml sterile, pre-labeled plastic straw. Most often one embryo is cyropreserved per straw.

4. Place straws horizontally in a freezing unit and cool from room temperature to -7°C at a rate of 5°C/min. Induce seeding at -7°C and freeze the embryo to -35°C at a rate of 0.5°C/min and plunge directly into LN2. Store straws in LN2 at -196°C.

b. Thawing Procedure.

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.

2. Cryopreservation of Goat and Sheep Embryos

a. Freezing Procedure.

1. 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.

2. Place one or two embryos between 2 air bubbles in a 0.25 ml sterile pre-labeled plastic straw.

3. Place the plastic straws horizontally in the freezing unit and cool from room temperature to -7°C at the rate of 5°C/minute. Induce seeding at -7°C and freeze embryos to -30°C at a rate of 0.3°C/min and then plunge straws directly into LN2. Store the straws in LN2 at -196°C.

b. Thawing Procedure.

1. Select the appropriate straw from the LN_2 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. Prepare the fasted recipient for a mid-ventral laparotomy. Once the uterus is exposed, transfer one or two embryos into the uterine horn(s) of each synchronized recipient female.

APPENDIX G. BOVINE OOCYTE COLLECTION PROCEDURES.

Appendix G1. Collection from dissected ovaries (Technique 1 - Slashing)

1. Materials

- Oocyte collection medium (OCM) – See Table G1
- L-Glutamine
- BSS + Heparin
- Pen/Strep
- 1x saline solution (0.9%) – See Table G4
- Petri dishes
- Bench top paper
- 400 ml beaker
- Scalpel handle
- Scalpel blades (sizes #11 and #20)
- Hemostat
- Mouth pipette (optional)

2. Preparations

a. OCM + Supplements (OCM+) – See Table G2

1. Prepare OCM+ by adding the following to one liter 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
2. Place OCM+ room temperature at least two hours before arrival of ovaries.

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

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

3. Procedures

a. Clean the ovaries with 1X saline solution

b. Slashing

1. Add 150 ml OCM+ to each beaker.
2. Attach a hemostat 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.

3. 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 #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.
4. Submerge the ovary into OCM and swirl it several times. Repeat this process until 10 ovaries have been processed/person.

c. Searching

1. 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.
2. 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.
3. Using aseptic technique, slowly aspirate OCM from the top of the beaker down to 50 ml. Be careful 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.
4. Transfer remaining media, with oocytes, to two grid plates. Wash the beaker with about 20 ml of OCM and add to the grid plates.
5. 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.
6. Transfer COC from first dish to the next leaving all debris behind (repeat twice to assure that oocytes are clean of debris).

Appendix G2. Oocyte Collection (Technique II - Aspiration)

1. Materials

- Holding Medium (HM) – See Table G5
- 1x saline solution (0.9%)
- Petri dishes
- Bench top paper
- 50 ml conical tube
- 10 ml plastic syringe
- 20-22 ga needle
- Mouth pipette (optional)

2. Preparations

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

1. Cover bench top with paper.
2. Add the following to the bench top:
   - 50 ml conical tube

- tube holder
- 10 ml air syringe
- 20-22 ga needle

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

3. Procedures

a. Clean the ovaries with 1x saline solution

b. Aspiration

1. Use the syringe to aspirate every follicle in the size range of 2-8 mm.
2. Deposit the follicular fluid slowly in the 50 ml conical tube
3. After aspirating all the ovaries allow the oocytes to settle.
4. Using Pasteur pipette, slowly aspirate the oocytes from the bottom of the 50 ml tube. Be careful not disturb the oocytes.
5. Place the oocytes in a grid dish containing enough holding medium (HM) to cover the dish.
6. Wash the oocytes with holding medium three times.
7. 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.
8. 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 G1. 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
Hepes (e.g. Sigma H-3375)	5.95 g	TC 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 liter. Sterile-filter medium into bottles. Store at 4^oC for up to 3 months. Date and label "OCM-Supplements". (TC = temperature controlled)

Table G2. 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 slashing – half of Pen/Strep and L-Glutamine aliquots can be refrozen.

Table G3. 10x Saline Stock Solution

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

Thoroughly mix ingredients. Sterile-filter. Date, label "10X Saline", and store at 4°C.

Table G4. 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 G5. Holding Medium (HM) – HEPPES 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 H. *IN VITRO* MATURATION OF BOVINE OOCYTES

1. Materials

- Petri dishes
- Mouth pipette (optional)
- Heat-pulled Pasteur pipette
- Oocyte Maturation Medium (OMM)
- 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).
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 I. 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 (optionsal)
- Heat-pulled Pasteur pipettes
- Sterile pipette tips and pipettor
- Microcentrifuge tubes (1.5 ml)
- Standard hemocytometer
- Centrifuge
- Sereomicroscope
- Medical grade mineral oil

2. Preparations:

1. Move PHE (400 µl) and Heparin (200 µl) from freezer to oven (39°C). PHE should be covered with aluminum foil (light sensitive).
2. Make fertilization microdrops
   a. 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).
   b. Make four 70 µl drops of FM in a 35 mm dish (washing medium). Cover with mineral oil.
   c. Equilibrate in CO_2 incubator (39°C) at least 2 hors.
3. Fill 1 conical tube with ~10 ml Hepes-Talp. Label tube.
4. Fill 1 conical tube with ~5 ml of IVF-Talp. Label tube.
5. Fill 1 conical tube with 5 ml SP-Talp. Label tube.
6. Also prepare 1 conical tube with 8mL Hepes-Talp.
7. Transfer tubes of HEPES-Talp (cap tightly) and SP-Talp (cap tight) to the water bath (39°C).
8. Transfer IVF-Talp (cap loose) to CO_2 incubator.
9. Prepare Percoll Gradient:
   a. Label 1 conical tube “Percoll Gradient” and fill the tube with 100 µl of Sperm-TL and 900 µl of ENHANCE-S Plus.

10. 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 Kimwipe 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 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 hemacytometer procedure)

1. 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.
2. Clean the hemocytometer and coverslip by washing with water followed by 70% EtOH; dry with a kimwipe.
3. Using a new pipette tip, transfer 10 µl of diluted sperm into each chamber (each side) of the hemocytometer
4. 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.
5. 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.
6. 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.

1. Calculations are based on the following parameters:
   a. 300 µl of final sperm suspension will be prepared
   b. 1 x 10^6 sperm/ml is desired in the final fertilization medium (this concentration can be adjusted if needed using Step 3 below)
2. 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 hemocytometer count (total hemocytometer count divided by 2)
3. Adjust for desired sperm concentration: 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 are 2 x 10^6 sperm/ml rather than 1 x 10^6 sperm/ml

   2 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.
4. Calculate volume of fertilization medium needed in the final sperm suspension: Substract the volume found in Step 3 from 300 µl from Step 1.
5. 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

1. 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.
2. Record time and date on each fertilization dish.
3. Incubate for 18 h at 39°C in a humidified atmosphere of 5% CO_2 in air.

M. Embryo Culture

1. 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.
2. 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.
3. Place the oocytes in the tube containing the hyaluronidase solution.
4. Vortex the oocytes at maximum speed for 3 min.
5. Transfer the oocytes to one 35 mm dish containing Hepes-Talp.
6. Wash the oocytes four times in CR1aa (1-3 days).

7. Place 10 oocytes in each drop of CR1aa (1-3 dys).
8. At day 3 transfer the oocytes in drops of CR1aa (3-7 days).
9. Check development at day 7.

4. Media Preparation

i. Sperm - TL

Ingredient	Final (mM)	mg/100ml	Location
NaCl (Sigma S-5886)	100	582	TC cabinet
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

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 ddH_2O before added to other ingredients.

Check for pH = ~7.4 and then adjust volume to 100 ml with double distilledH_2O.

Sterile-filter into a bottle.

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

ii. IVF-TL

Ingredient	Final (mM)	mg/100ml	Location
NaCl (Sigma S-5886)	114	666	TC cabinet
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

Add NaCl, KCl, $NaHCO_3$, NaH_2PO_4, Lactic acid, and Phenol red into a beaker. Bring volume to 80ml 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 100ml with ddH_2O. Sterile-filter into a bottle. Date, label "IVF-TL", and store at 4°C for 2 weeks.

iii. HEPES-TL

Ingredient	Final (mM)	mg/500 ml	Location

NaCl (Sigma S-5886)	114	3330	TC cabinet
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

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.

iv. 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 l	refrigerator
Pen/Strep (Gibco 15140-122)	100 l	freezer

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

v. 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.

vii. 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 l	freezer

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

vii. 10X SP-TL (for percoll gradient)

Ingredient	g/100 ml	Location

NaCl (Sigma P-5886)	4.675	TC cabinet
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

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^oC one month.

APPENDIX J. HARVESTING SAMPLES FOR DNA EXTRACTION

1. Blood. It was recommended that the volume of blood for DNA extraction should be split between two tubes at the time of collection. This served to reduce the risk of the accidental loss of all the sample from an individual. It is recommended that the samples remain split through processing and storage; furthermore, the processing of the two halves should be carried out in different batches. Thus, if an unforeseen failure of the extraction technique occurs in one batch, DNA from the individuals affected by the failure can still be obtained from the other half.

Once extracted, DNA should be put into labeled aliquots of 50 µl with a concentration of 200 µg/ml before long-term storage. This division will avoid repeated freezing and thawing. The DNA may be safely stored at 4°C over 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, place in a -20°C temperature or lower (LN2) but the latter is not necessary.

2. Somatic Cells. DNA can be extracted from many different animal tissues, although skin and hair follicle cells are the easiest to collect under flied conditions. Once the samples are obtained they may be stored at 4°C before DNA extraction. The procedures for DNA extraction are technically demanding. The procedures should not be used as part of a conservation program until the capability of the laboratory concerned has been established. This should be done by demonstrating successful extraction of DNA, with the anticipated yields, from samples that are of the same tissue and species as those being obtained as part of the program.

Some chemicals used in the procedures should be treated with care, and attention should be given to the health and safety information that should accompany all laboratory products. However the procedures for DNA extraction can be safely undertaken in well-managed and technically experienced laboratories.

APPENDIX K. ARTIFICIAL INSEMINATION PROCEDURE FOR CATTLE

1. Equipment and Supplies Needed for AI in Cattle.

A. Equipment:

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

B. Renewable Supplies:

- Boots (washable)
- Coveralls (washable or disposable)
- Plastic gloves (disposable)
- Plastic foot covers
- Paper towels
- Disinfectant liquid
- Liquid soap
- Scissors
- Semen (1 unit per cow)
- Plastic straws for the embryos (e.g., 0.25 ml)
- LN2
- Record book (Important!)

2. Detecting Estrus for Artificial Insemination in Cattle

Estrous detection is one of the most important in-field procedures to having a successful AI program. Obtaining a high rate of successful AI will maximize the use of the stored germplasm in the gene bank.

A well-trained person should check for standing estrus (heat) in pasture-roaming cattle for at least 30 minutes early in the morning (as early as possible after sunrise) and again for a minimum of 30 minutes just prior to sun down. It is highly recommended to detect estrus in each female group at least twice daily for a successful AI program. Some breeding operations have begun checking the herd for estrual behavior a third time at between 10:00 and 11:00 PM at night to improve their detection efficiency.

The more females in the breeding group the longer the observer should evaluate the herd. For cows that are in standing estrus or suspected of being in estrus, the following notations for that date (AM or PM) are recommended to be placed in the respective female's records:

AM or PM

S—Standing Estrus

R—Riding

A—Active

M—Mucus on vulva

Also the name or initials of the observer and the correct date should be noted.

3. Artificial Insemination Procedure.

1. It is recommended to artificially inseminate cattle after restraining them in a cattle working stocks or chute. For cryobank semen, at least one unit of semen should be warmed and evaluated for sperm quality prior the planned insemination.

2. Following restraint, animal identification number or code letters need to be verified and the proper semen thawed following instructions provided by the semen company. Information to be recorded should include semen code for each animal inseminated. Immediately following thawing of semen, the semen straw is placed into an insemination gun (e.g., 0.25 ml Cassou gun/ apparatus) and the sheath properly placed over the insemination gun again following manufacturer guidelines. Following loading of the insemination gun, the proper temperature should be maintained (i.e. out of direct sunlight) until the insemination gun is inserted into the vagina.

3. Following loading of the insemination gun, the inseminator will place an arm-length disposable plastic glove over their palpation hand and arm and apply sufficient lubrication (most often a commercial carboxymethylcellulose gel product). The inseminator will place their arm into the female's rectum and locate the cervix (below) by palpation downward at the ventral rectal wall.

4. Following location of the cervix, the inseminator should then clean the external vulva area of the animal with clean disposable paper towels to help remove any mucus, dirt and fecal material. This procedure is recommended to help prevent contamination of the insemination gun and the reproductive tract during insemination.

5. Upon cleaning of the vulva area, the inseminator gently guides the insemination gun into the vagina (the distal tip pointing upward) through the cervical canal and into the body of the uterine to deposit the thawed semen. The last portion of the semen in the unit is often deposited in the cervical canal while removing the insemination gun. Minimum manipulation should be used during AI to avoid to injury the endometrium, which could reduce the chances of a pregnancy. The ease of insemination should be assessed recorded by the technician (e.g., 1 = good, 2 = moderate, 3 = difficult)

6. Following completion of semen deposition, the animal should be quietly released from the working chute and slowly returned to their assigned pen. If the female is still nursing a calf at the time of AI and the calf has been removed for the AI procedure, the calf should be returned to the female and allowed to nurse before she is returned to the pasture.

APPENDIX Λ. CERVICAL ARTIFICIAL INSEMINATION PROCEDURES FOR SMALL RUMINANTS

1. Equipment needed

- Breeding stand or facilities to restrain the doe
- Paper towels
- Speculum (25 x 175 mm to 25 x 200 mm for does)
- Vaginal lubricant (non-spermicidal)
- AI tank and semen
- Thawing box
- Thermometer
- Straw cutter
- Insemination gun

AI protocols for sheep and goats are similar. The following was adapted from a protocol for goats http://www.aces.edu/pubs/docs/U/UNP-0095/UNP-0095.html).

2. Preparing Does and Ewes for Artificial Insemination

The female must be in good health status and have sufficient body condition prior to breeding. If necessary and possible, the ration should be supplemented with concentrate feeds and minerals for a 30-day period prior to insemination. Determine if does will be bred on normal or synchronized heat.

3. Estrous synchronization:

Several hormone protocols that have been recommended for estrous synchronization in goats: progesterone or prostaglandins F2 alpha ($PGF_{2\alpha}$). The choice and efficacy of the method depend on the season and availability of the injectible agents. Prostaglandins work only if does are cycling regularly and have a functional CL. Progesterones are thus recommended for breeding out of season or during anestrous periods. Different countries will have different regulations on the use of these agents.

4. Protocols for estrous synchronization of does and ewes using a progesterone-releasing intravaginal device

PROTOCOL ONE

Day 1: Insert progesterone-releasing intravaginal device (CIDR) with appropriate applicator.

Day 7 or 17: Administer 1.5 cc (ml) of PGF_2 intramuscularly.

Administer intramuscularly 2.9 cc (ml) of PG 600 = drug consisting of 400 IU of Pregnant Mare Serum Gonadotropin (PMSG) and 200 IU of Human Chorionic Ganadotropin (HCG).*

Day 8 or 18: Remove the CIDR.

Place the female together with a teaser animal and begin regular estrous detection. Upon detection of estrous, the doe should be inseminated once (at 18 hours post-detection) or twice (at 18 and 24 hours). As noted in Section 11.4.1, a teaser is a vasectomized male (or a doe treated with estrogens or androgens). Use of a harness marker that leaves a colored sign of mounting will improve detection of mounting.

If labour is not available for regular estrous detection, the doe or ewe can be bred “on-appointment”, 48 (ewes) to 54 (does) hours after removal of the CIDR. Conception rate may be

decreased by 30 to 50% when breeding by appointment is performed, relative to breeding after estrous detection.

PROTOCOL TWO

Day 1: Insert CIDR with appropriate applicator.

Day 8 or 18: Remove the CIDR.

Administer intramuscularly 1.5 cc of PGF_2 and 2 cc of PG 600.

Inseminate the female after heat detection or by-appointment as described for Protocol One.

During the breeding season, the use of the gonadotropin (i.e. PG 600) is not needed. However, placing the female together with the teaser animal immediately after removal of the CIDR will improve heat expression, detection and ovulation and, therefore the efficiency of AI.

5. Estrous detection

The female should be checked for estrous at least twice per day, in the early morning and late afternoon.To identify heat, observe the ewe or doe's behavior and examine the external genitals for redness, swelling or a mucous discharge. Once heat is identified, the time of onset of estrus shuld be recorded and monitoring should continue.

6. Performing intrauterine AI

1. The use of a breeding stand will facilitate AI. The female should be placed in the stand with the back legs up, so as to elevate the back allow the front legs to provide support with the neck and head facing toward the ground.

2. If needed, wash and dry the vulva to remove any dirt.

3. Introduce the vaginal speculum, using a lubricant if necessary. Be sure to use an appropriately-sized speculum. To introduce the speculum, open the labia of the vulva with one hand and with the other hand, gently introduce the thinner extremity of the speculum. Once introduced in the vagina, light pressure can be used to orient the speculum toward and down to the vaginal floor. An auxiliary light source may help one to visualize the cervical os and to distinguish it from the pleats of the vagina.

4. Remove any excessive vaginal mucus with the speculum.

5. Remove the desired semen straw from the LN2 tank and thaw the straw semen in water of 37°C. Take care to not remove other straws from the same canister of the tank and to not expose the straw to direct sunlight.

6. Warm the barrel of the straw gun.

7. Remove the straw from the water bath and dry with a clean paper towel.

8. Cut the correct extremity of the straw and insert into the AI gun as quickly as possible to avoid temperature changes.

9. Place the plastic sheath over the gun barrel.

10. Return to the animal and introduce a clean vaginal speculum, again removing excess mucus.

11. Introduce the gun into the vagina, passing through cervical rings until it reaches the interior of the uterus. If resistance is encountered, deposit the semen in the exterior of cervix, and make a note of in the AI recordbook.

12. Remove the gun and speculum, leaving the female on the stand for a few minutes in the standing position.

13. Check for abnormalities such as reflux of the semen into the gun or semen left in the straw.

14. Release the female and record the AI information.

7. Record keeping

Records should be kept of the following information:

- Identification number or name
- day and time of implantation of the CIDR
- day and time of heat detection
- date and time of AI
- AI technician
- ID number and breed of the male
- straw identification by date when semen was frozen and processor

8. Factors affecting success of AI

1. female's reproductive soundness, general condition, and nutritional status
2. efficiency of estrous detection and the method of estrous synchronization
3. timing and the number of AIs performed

9. Laparascopic Artificial Insemination

As an alternative to cervical AI (particularly for sheep), semen can be deposited directly into the uterus, via the surgical technique of laparoscopy. In laparoscopic AI, the semen is deposited into one or both uterine horns from a sharp-tipped glass pipette or needle and syringe inserted through the ventral wall of the abdomen. This technique can be applied with much smaller sperm doses than with cervical AI, which may be attractive for particularly valuable semen from a gene bank. Although the technique is only minimally invasive, laparoscopic AI should only be performed by a veterinarian or highly-trained technician, however.

APPENDIX M. PREGNANCY RATES FROM ARTIFICIAL INSEMINATION AND SURGICAL INSEMINATION IN FARM ANIMALS WHEN USING FROZEN-THAWED SEMEN

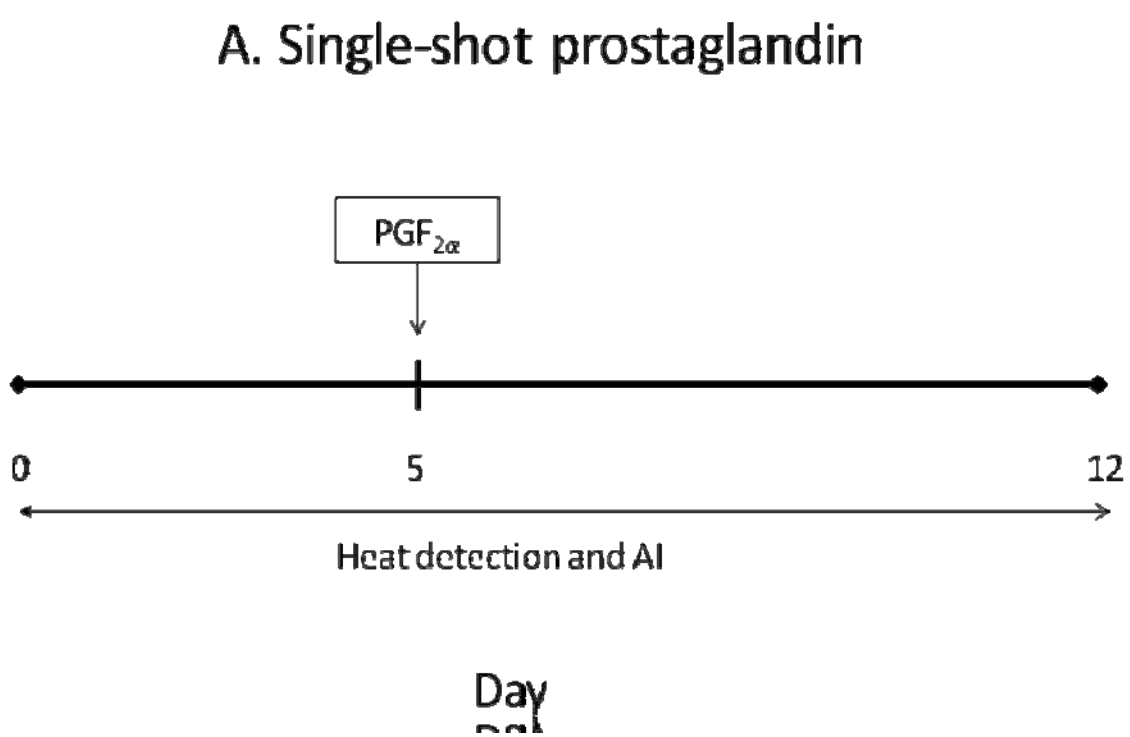
Parameter[a]	Cow	Sheep	Goat	Sow	Mare
Pregnancy success with AI (%)	45-75	15-50	20-55	60-90	35-70
Difficulty of the AI procedure[b]	1	5	4	2	1
Recommended use of AI	yes	yes	yes	yes	yes
Pregnancy success with surgical AI (%)	65-85	55-85	55-85	85-90	60-80
Difficulty with surgical AI	4	1	1	2	3
Recommended use of surgical AI	no	yes[c]	no	no	no

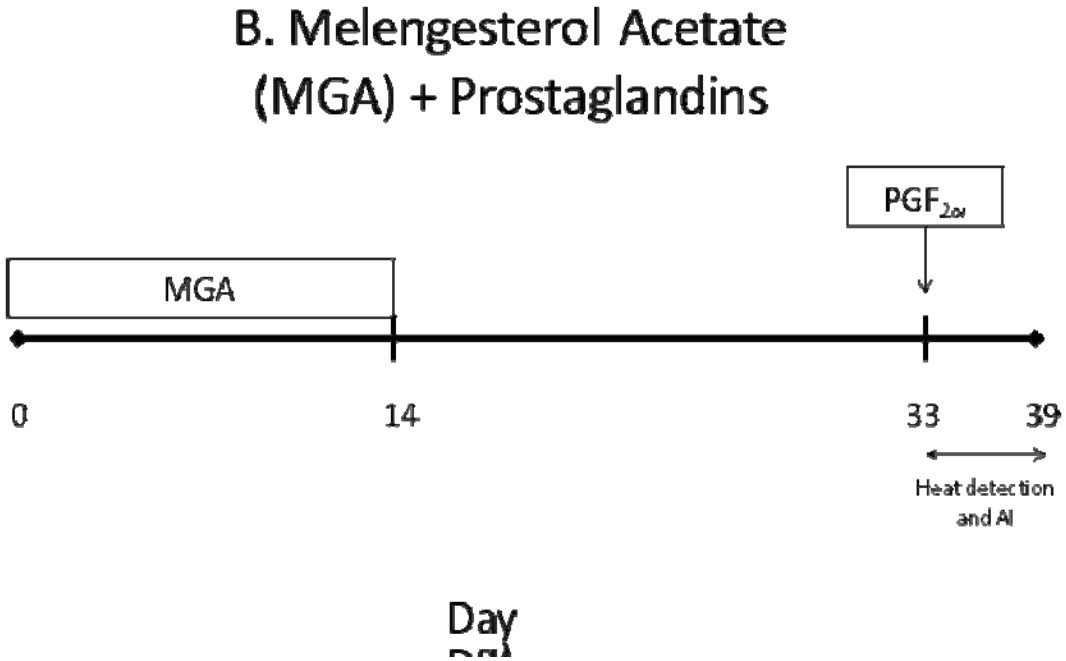
[a] Ranges presented are estimated from multiple scientific and in-field sources. The values vary based on expertise of the technicians, optimal animal nutrition and management practices.

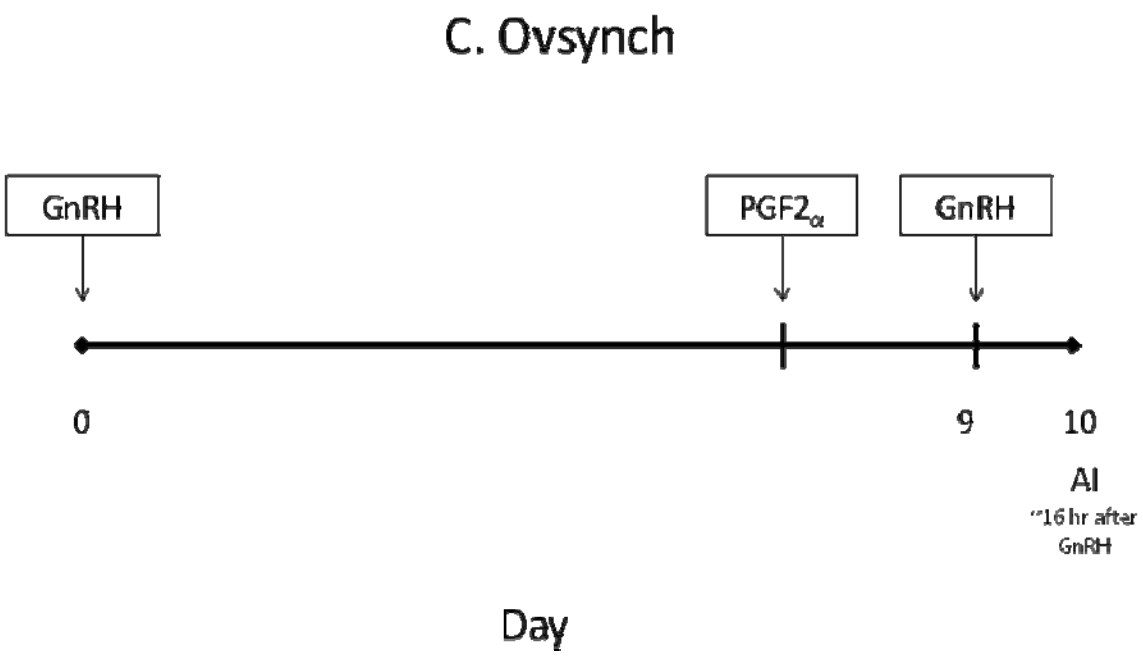
[b] Difficulty of the insemination procedure with 1 being the easiest and 5 being the most difficult to perform.

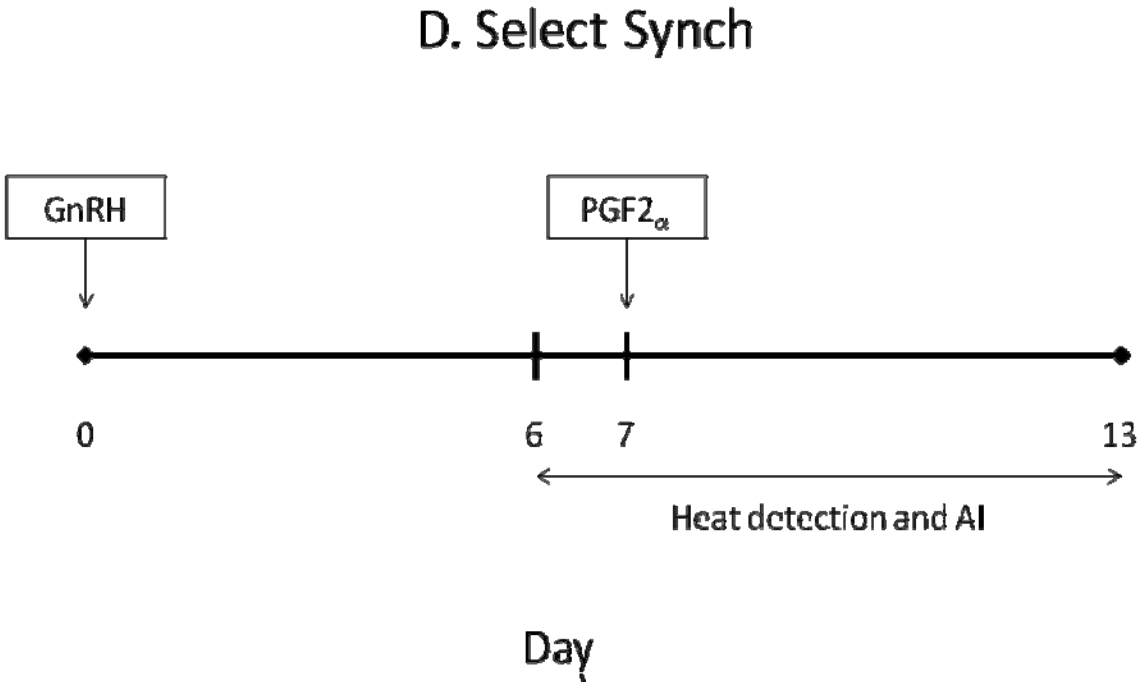
[c] assuming that experienced surgical team is available to conduct the procedure.

APPENDIX N. COMMONLY USED ESTROUS SYNCHRONIZATION METHODS FOR CATTLE

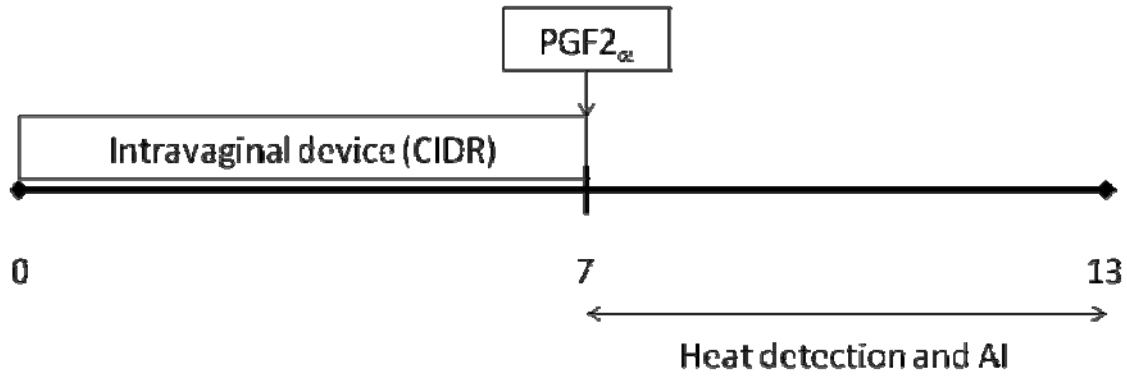




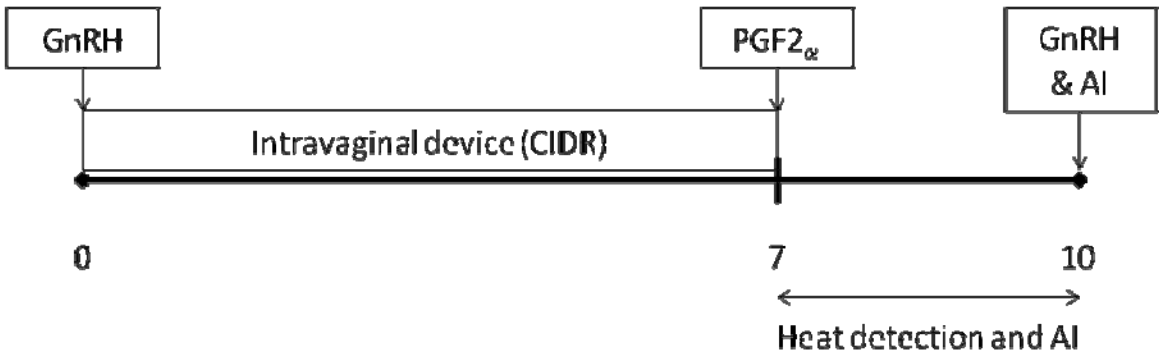

C. Ovsynch
GnRH
PGF2α
GnRH
0
9
10
AI
*16 hr after GnRH
Day


D. Select Synch
GnRH
PGF2α
0
6
7
13
Heat detection and AI
Day

E. CIDR and prostaglandins



F. Select Synch with CIDR



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