ILRI MANUAL

53

Laboratory training manual on biobanking and recovery of indigenous poultry genetic resources by cryopreservation of primordial germ cells (PGCs)

In collaboration with the
Centre for Tropical Livestock Genetics and Health


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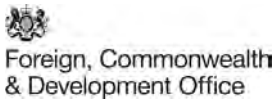
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Laboratory training manual on biobanking and recovery of indigenous poultry genetic resources by cryopreservation of primordial germ cells (PGCs)

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CGIAR is a global partnership that unites organizations engaged in research for a food-secure future. The CGIAR Research Program on Livestock provides research-based solutions to help smallholder farmers, pastoralists and agro-pastoralists transition to sustainable, resilient livelihoods and to productive enterprises that will help feed future generations. It aims to increase the productivity and profitability of livestock agri-food systems in sustainable ways, making meat, milk and eggs more available and affordable across the developing world. The Program thanks all donors and organizations who globally supported its work through their contributions to the CGIAR Trust Fund.

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The Manual details the content of advanced research and technology transfer from CTLGH to technical staff of African Member States and regional gene banks in collaboration with the African Union InterAfrican Bureau for Animal Resources (AU-IBAR)

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Acronyms

ABS	Access and benefit sharing
ABS-CH	Access and benefit sharing clearing house
AnGR	Animal genetic resources
AnGR-TAG	Animal genetic resources taxonomy advisory group
AU	African Union
AU-IBAR	African Union – InterAfrican Bureau for Animal Resources
BRL	Buffalo rat liver
CTLGH	Centre for Tropical Livestock Genetics and Health
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EMA	Embryonic mouse antigen
ES	Embryonic stem
ETOH	Ethanol
FACS	Fluorescent-activated cell sorting
FAOT	FGF2, Activin A, ovotransferrin
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FKBP	KK506 Binding protein
GFP	Green fluorescent protein
HH	Hamburger and Hamilton

ILRI	International Livestock Research Institute
IRCC	Internationally recognized certificate of compliance
IUCN	International Union for Conservation of Nature
MAT	Mutually agreed terms
NAPRI	National Animal Production Research Institute
NARS	National Agricultural Research System
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGC	Primordial germ cells
PIC	Prior informed consent
RPM	Revolutions per minute
SASSO	Sélection Avicole de la Sarthe et du Sud Ouest or Poultry selection from Sarthe and Southwest
SSEA	Stage-specific embryonic antigen
STO	Sando inbred mouse derived thioguanine-resistant and oubain-resistant
TPZ	Tirapazamine
UK	United Kingdom

1. The rationale and relevance of the training manual

1.1 Rationale

Preservation of poultry breeds or lines is a challenging process, especially if it is done through cryopreservation. This is due to the unique structure of the avian ovum compared to that of mammals and the poor fertility of frozen poultry semen. In addition, cloning using somatic cell nuclear transfer is not an option because embryo transfer in avian species is not feasible. However, recent developments at the Centre for Tropical Livestock Genetics and Health (CTLGH) (The Roslin Institute, University of Edinburgh, United Kingdom), indicate that isolation and freezing of primordial germ cells (PGCs) may provide an alternative approach for biobanking of poultry germplasm.

Primordial germ cells are specialized stem cells that will eventually differentiate into sperm or ova depending on the sex of individual embryos. The PGCs can be isolated from the blastodisc (4–6 hours after fertilization), blood (day 2–3 embryos) and gonads (day 9 embryos). Following isolation, PGCs can be cultured or cryopreserved and later thawed for transfer into Day 2–3 recipient chick embryos. Transferred PGCs will then develop and differentiate into gametes (ova or sperm) alongside the recipient bird's own PGCs. The resulting gametes will, therefore, be derived from both the donor and the recipient breed thus facilitating the regeneration of the breed (donor) that provided the cryopreserved or cultured PGCs. The chicken produced from this process is called a chimera. Furthermore, the recovery of cryopreserved PGCs could be enhanced by using genome-edited sterile birds as recipients. The use of a sterile surrogate resolves the problem of competition between PGCs from the donor and the recipient, which represents a significant milestone in biobanking techniques. These developments offer exciting possibilities for the conservation of poultry breeds.

Current research on cryopreservation of PGCs has focused largely on the chicken. Therefore, it will be necessary to establish how and under what conditions, this approach can be applied to other poultry species. While the technology is invaluable in the preservation of poultry 'breeds at risk', it may also play an important role within poultry breeding companies. For instance, it could be used to maintain important parental lines of mainstream poultry breeds used in commercial poultry production without the need to keep large populations of live birds.

The transfer of such technology and practice to the National Agricultural Research Systems (NARS) in Africa may solve problems faced by the local poultry sector. Indeed, north-south and south-south knowledge exchange can partly address challenges pertaining to lack of information and capacity building for both scientists and professionals while facilitating the scaling up of successful solutions. Therefore, the CTLGH-ILRI Reproductive Technology and Precision Breeding program is designed to assist African countries enhance chicken productivity while preserving genetic resources. In collaboration with the African Union – InterAfrican Bureau for Animal Resources (AU-IBAR), the program focuses on capacity building for cryoconservation of African poultry genetic resources.

Mastering methods for harvesting and in vitro conservation and propagation of chicken PGCs is a valuable step in understanding the biology of PGCs. The ability of chicken PGCs to form functional gametes after long-term culture can be

used to develop a cell-based system for genetic improvement of the chicken genome. Stem cells have been a key tool in avian biotechnology and are presently seen as one of the most promising tools for maintaining avian genetic biodiversity across Africa without moving germplasm from their regions of origin.

This manual was designed following requests from the NARS and the regional gene banks established under the AU-IBAR projects. It provides an opportunity for experts in poultry biotechnology, managers of animal gene banks, the regional and Sub-regional Focal Point for Animal Genetic Resources in Africa and members of the AU Animal Genetic Resources Taxonomy Advisory Group (AnGR-TAG), to improve their capacities and acquaint themselves with techniques in breeding and conservation of poultry genetic resources. Users of this manual will be acquainted with various techniques used in chicken PGCs cryobanking and transfer. These techniques range from isolation of PGCs from various biological materials, cryopreservation and thawing, viability assessment of cultured PGCs, re-injection into the blood circulation of a developing embryo and evaluation of the integration ratio of the injected cells in the gonads.

1.2 Relevance

Most poultry genetic resources are maintained in living populations (in situ). However, conserving genetic resources in situ always carries the risk of loss due to pathogen outbreaks, genetic problems, breeding cessation and/or natural disasters. Cryobanking of germplasm in birds has historically been limited to the use of semen, thus preventing conservation of the female W chromosome and mitochondrial deoxyribonucleic acid (DNA). As an alternative, avian PGCs, the first germ cell population established during early development, are incorporated into the gonads (Yasuda et al. 1992) where they can differentiate into functional gametes following transplantation to recipient embryos (Tajima et al. 1993; Ono et al. 1998). A further challenge is posed by the structure of avian eggs, which restricts the cryopreservation of ova and fertilized embryos, a technique widely used in mammalian species. By using a unique biological property and accessibility of these stem cells (precursor cells for gametes, which temporarily circulate in the vasculature during early development), an avian PGC transplantation technique has been established. To date, several techniques for PGC manipulation including purification, cryopreservation, depletion and long-term culture have been developed in chickens. Primordial germ cell transplantation combined with recent advanced PGC manipulation techniques have enabled ex situ conservation of poultry genetic resources in their complete form. In this manual, the updated technologies for avian PGC manipulation are introduced and the concept of a poultry PGC bank is proposed by considering the biological properties of avian PGCs.

2. Introduction to germplasm cryobanking

A rapid and constant decline in animal species populations has been reported in recent years (IUCN Species Survival Commission 2010). While in situ/in vivo conservation strategies are still very relevant in the preservation of animal biodiversity, other strategies such as germplasm (reproductive cell) cryopreservation are essential. Germplasm is a live information source for all the genes present in respective animal species, which can be conserved for long periods and regenerated for future use whenever required. Germplasm cryobanking or cryoconservation is the most successful method to conserve the genetic traits of endangered and commercially valuable species. This is an attractive addition to the indispensable maintenance of genetic diversity through rearing of live animals. However, research is needed to effectively apply these techniques in a multidisciplinary program aimed at the preservation of the numerous subpopulations of animals that exist within a given species.

Germplasm cryobanking involves the harvesting and freezing of gametes, embryos, gonadal tissues, somatic tissues or PGCs of species threatened with extinction. There is considerable diversity in cryobiological characteristics among cell types and tissues of each species. Research done by scientists from the Centre for Tropical Livestock Genetics nd Health (CTLGH) has focused on developing techniques for the conservation and recovery of the genetic material of the chicken. This training manual will describe the techniques and potential use of cryopreserved PGCs for research, development and restocking of poultry populations.

3. Local chicken breeds of Africa: their description, uses and conservation methods

3.1 Some definitions

Breed: An established group of birds within a species related by breeding, possessing a distinctive shape, conformation, plumage colour, comb type, general body weight and breeds true. Examples include Shika Brown, Venda, Ovambo, FUNAAB Alpha Potchefstroom Koekoek, Tswana, Boschveld etc.

Class: Used to designate a group of birds developed in certain regions or geographical areas. Some examples are American, Asiatic, Mediterranean and English.

Cryobiology: Study of the effects of low temperatures and freezing on organisms (often for the purpose of achieving cryopreservation).

Cryogenics: A discipline within physics. It is the study of the production and behaviour of materials at cryogenic temperatures.

Cryopreservation: The process of preservation or conservation of biological materials at cryogenic temperatures (usually lower than –180°C).

Germplasm cryopreservation: The cryopreservation of genetic materials of germ cells, such as seed, sperm, or eggs.

Lines: Subclasses of a strain developed such that the gene(s) responsible for a particular trait is fixed to be utilized for production of commercial hybrids.

Strain: Subclassification of a breed. Normally a strain is named after the person who developed it or the institution where it was developed. Strains are developed by emphasizing specific traits like egg production, early maturity, better feed efficiency and egg weight etc. Examples are the KALRO KIC1 and KIC2

Variety: Term used to subclassify breeds. There may be many varieties within a breed differentiated by plumage colour, pattern and comb type, e.g. White Leghorn, Black Leghorn, Brown Leghorn and Barred Plymouth Rock etc.

Vitrification: The cryopreservation of biological materials at an extremely high cooling speed without ice crystal formation.

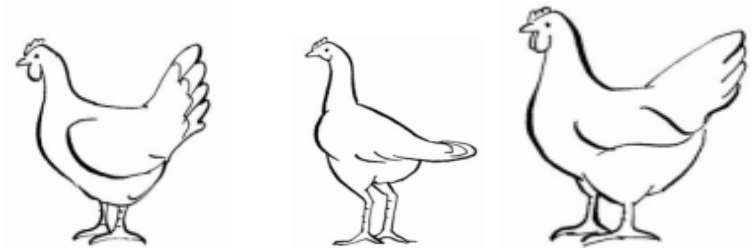
3.2 General characteristics of the African chicken

In the African context, indigenous poultry refers to local breeds of poultry that are adapted to harsh environmental conditions. They are often reared in extensive, small-scale village, free range and organic production systems. They are also referred to as traditional, scavenging, backyard, village, local or family poultry. These breeds exhibit the following characteristics:

- Hardy and rustic, showing adaptation to harsh environmental conditions
- Self-reliant, scavenging for kitchen waste, insects, worms, lizards, plant seeds and leaves etc.
- Small with slow growth rates
- Meat and eggs have attractive pigmentation, low fat and good sensory attributes
- Good brooding aptitudes
- Age at sexual maturity is between 133-169 days under extensive production systems
- Poor egg production (40-50 eggs/year under an extensive management system)
- Good fertility and hatchability rates

These chickens are genetically adapted to harsh environments characterized by limited feed and water resources, poor weather and climatic conditions and exposure to pathogens and predators. The chickens are also genetically diverse, requiring minimum inputs to produce meat and eggs for local and international markets. There are also several locally improved breeds, which can support commercial production systems in Africa. The orientation for development and/or production could roughly be guided in rural settlement by the morphological characteristics of the animals (Figure 1).

Figure 1. Morphological features of various types of chickens.



Egg types Meat type Dual purpose

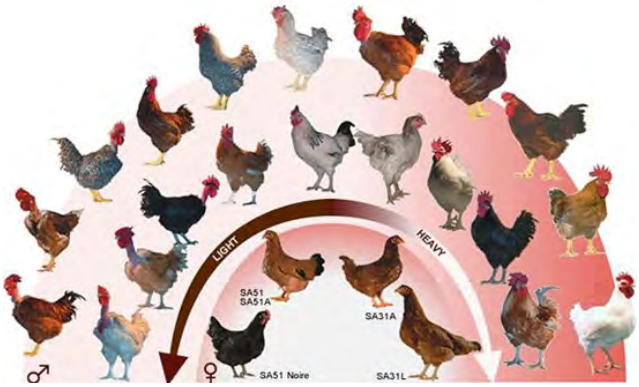
source: TECA-FAO, 2010. For reference section TECA - Technologies and Practices for Small Agricultural Producers, FAO, 2010. Small-scale chicken production. https://teca.apps.fao.org/teca/en/technologies/6949. consulted 08/12/2021

Below are some of the most promising indigenous and locally adapted chicken breeds found in Africa (Figure 2 and Table 1).

Figure 2. A breeding family of Kabir chicken in Cameroon.

Source: GreenGold AgroVenture Cooperative Society, Cameroon.

Table 1. Selected improved indigenous chicken breeds found in Africa

SASSO family of breeder roosters and hens	 source: Brigido LaGuardia, 2021. For rerefence section Brigido LaGuardia, 2021. What Is SASSO Chicken. in Agriculture and Livestock Guide. https://putakputak.com/poultry/chickens/what-is-sasso-chicken/. consulted on 08/12/2021	The Sélection Avicole de la Sarthe et du Sud Ouest or Poultry Selection from Sarthe and South West (SASSO) chicken breeds are very popular in Africa due to their ability to withstand local poultry-rearing conditions. The popular lines of the breed are the SASSO Rainbow T (TR), SASSO Rainbow X (XR), SASSO Ruby C (C44), SASSO Ruby N (XL44N) and SASSO Ruby T (T44).
		These chicken strains are developed by SASSO, a poultry breeding company in France. They are becoming popular for use in backyard and large-scale free-ranging operations in Africa.
Kuroiler	 Source: Kuroiler Chicken Farmers in KENYA. https://www.facebook.com/KuroilerKenya/	The Kuroiler is a composite breed derived from crossing either coloured broiler males with Rhode Island Red females or White Leghorn males with female Rhode Island Reds. Developed by Kegg farms in India, this dual-purpose breed can live on a diet of kitchen and agricultural waste and produces around 150 eggs per year. The meat yield per bird of Kuroilers is also greater at 3.5 and 2.5 kg for males and females, respectively. The Kuroiler is resistant to diseases[1].
Shika Brown	 source: African Chicken Genetic Gain (ACGG-ILRI)	The Shika Brown chicken breed is the preferred layer strain in Nigeria. It was created by the National Animal Production Research Institute (NAPRI) at Ahmadu Bello University in Shika, Zaria state of Nigeria. Its characteristics include high production rate, persistency, livability and feed conversion efficiency. The chicken produces large size eggs with excellent shell quality. The Shika Brown is well adapted to the harsh tropical environment and is resistant to many diseases of economic importance[2].

1 https://en.wikipedia.org/wiki/Kuroiler

2 *ACGG fact* sheet 1, 2016

FUNAAB Alpha	 Source: African Chicken Genetic Gain (ACGG-ILRI)	The FUNAAB Alpha is a dual-purpose chicken breed developed by the Federal University of Agriculture, Abeokuta, Ogun state of Nigeria. FUNAAB Alpha attains body weights of 2.6 and 1.8 kg at 20 weeks of age for males and females, respectively. Egg colour, weight and number were improved from white to brown, 39 to 55 g and 120 to 250 per annum, respectively.
Noiler	 Source: African Chicken Genetic Gain (ACGG-ILRI)	The Noiler is a dual-purpose chicken breed developed by the Amo Farm Sieberer Hatchery Limited, Awe, Oyo state in Nigeria. The Noiler is a hybrid of a broiler and cockerel, therefore, possesses desired traits of both a broiler and cockerel. Unlike broiler chickens, Noiler chickens come in varying colours that include black, white, yellow, brown and grey patches.
Fulani	 Source: African Chicken Genetic Gain (ACGG-ILRI)	Originally kept by the Fulani people, the Fulani ecotype chickens are aggregated and multiplied by the Obafemi Awolowo University, Ile-Ife, from a base population common in the northern region of Nigeria. They are good egg layers with high meat quality. These chickens are disease tolerant and tend to live long.

Different breeds of African chickens may have variable performances as shown in Tables 2 and 3 below. Some ecotypes of chicken breeds in Africa have high genetic potential that is, unfortunately, not fully or efficiently utilized.

Table 2. Some performance parameters of cocks at 20 weeks of age

	Fulani	FUNAAB Alpha	ShikaBrown	Kuroiler	Sasso	Noiler
						
Live weight (kg)	1.3	2.1	1.7	2.9	3.0	2.6
kg Feed/kg body weight	8.5	5.2	7.0	4.6	5.4	5.7
Protein (g/kg meat)	114	269	158	320	348	213
Fat (g/kg meat)	11	29	15	35	42	15

Source: African Chicken Genetic Gain (ACGG-ILRI)

Table 3. Some performance parameters of indigenous and locally adapted hens

	Fulani	FUNAAB Alpha	ShikaBrown	Kuroiler	Sasso	Noiler
						
Age at 1st Egg (weeks)	18	17	17	18	19	17
Ave Egg Weight (g)	42	51	54	55	55	39 (at 1st month of lay)
No. of Eggs/Week (3rd month of lay)	3	4	5	4	3	2 (2nd month of lay)
Chicks Hatched/100 Eggs	60	55	74	81	85	84

Source: African Chicken Genetic Gain (ACGG-ILRI)

Some composite dual-purpose breeds like the Kabir (figure 2) have attracted a lot of attention of many farmers in some regions of Africa, thanks to their hardiness and productivity in scavenging and semi-intensive systems.

Figure 2. Kabir chicken from the GreenGold agroventure farm in Cameroon.

African farmers also keep some locally adapted exotic strains of chickens to produce meat and/or eggs. These include Vedette, Rhode Island Red, Plymouth Rock, New Hampshire, Orpington, Brahma, Cornish, Leghorn, Sussex and Flaveroles, among others. Below are some locally adapted exotic chicken breeds that are used for village and commercial production in Africa (Table 4).

Table 4. Some locally adapted exotic strains of chickens used for meat and/or eggs in Africa

Sussex Sussex chickens are dual-purpose birds that produce about 250 eggs of varying shades of light brown annually. Their plumage comes in eight different colours, the most common being the pure white body with a black neck and tail feathers. They are very calm and are comfortable within free range systems.	
Rhode Island Reds Rhode Island Reds are dual-purpose chickens, meaning they can be raised for their eggs or meat. They are one of the most popular backyard and commercial chicken breeds because they are hardy and produce many eggs. This breed can produce about 250 brown, medium sized eggs a year. They have brown and black feathers giving them a dark appearance.	
Plymouth/Barred Rock They are predominantly grey with white stripes around their bodies. Barred, black frizzle, blue, partridge, buff, Columbian, silver pencilled, black and white are common colours in this breed. They are better suited for the free-range system and are very friendly and hardy. The hen produces about 280 light brown eggs a year.	
Australorp The Australorp breed is one of the most productive chicken breeds and originates from Australia. It is raised for both eggs and meat because it is a very good layer and is also hardy. Australorp produces about 250 brown eggs per year. They forage very well and come in black, white or blue colours.	
Wyandotte The Wyandotte is a dual-purpose breed and produces about 200 brown eggs annually. They have a docile temperament. They have attractive plumage with gold, blue and silver colour variations.	

Jersey Giant The Jersey Giant chicken breed is dual-purpose and the hen produces about 260 brown eggs a year. Jersey Giant chickens are large-framed. These blue, black and white chickens are the largest of the purebreds. They have a docile temperament and are excellent for beginner flocks.	
Leghorn Leghorn chickens are productive layers. The hen produces about 280 white eggs a year. They are not of a docile temperament. They are rather flighty and startle easily. However, they are very productive layers. They thrive particularly well in warmer climates.	
Orpington The Orpington is an attractive and friendly dual-purpose breed. They are good layers and are hardy. The Orpington birds are fluffy and have multiple colours, such as blue, buff, black, white and lavender.	
Barnevelder The Barnevelder is native to Holland, and it is a cross between the Dutch Landrace and Asian jungle fowl. A Barnevelder hen produces around 200 eggs per year. The eggs are small to medium in size and are light speckled brown in colour. This breed is mostly black with brown tipped feathers. It is better suited for the backyard. It is not a good flyer, therefore, there is no need to clip its feathers.	
Marans Marans look like Plymouth Rocks and are mostly dark grey with white flutters. Marans are medium-sized, dual-purpose birds that produce about 200 eggs a year. They are renowned for their vibrant dark brown and tasty eggs. Their eggs are usually medium-sized. Marans are gentle and do not require a lot of space to roam. However, they are not of a docile temperament and, therefore, do not make good pets.	

4. Poultry germplasm cryopreservation

In general, African avian breeds are imperiled. The progressive loss of this genetic diversity is caused by industrial consolidation of exotic breeds, combined use of highly effective genetic selection and reproductive technologies and changing economic conditions. In addition, intense selection for economically desirable traits, results in genetic abnormalities or genetic combinations that lower viability and profitability. Addressing this loss of genetic diversity requires alternative genetic stocks. Successive outbreaks of avian influenza have demonstrated how easily genetic resources can be lost and leading to long- and short-term economic losses. Diverse genetic resources are needed to develop lines that are resistant to emerging diseases and resilient to changing climate. Biobanking of African poultry genetic resources will allow researchers/breeders to quickly mitigate threats to local production systems. Reserves of poultry genetic material provide protection to African producers and consumers. Conservation of poultry genetic resources also provides breeders with the resources needed to structure local populations to meet the growing global demand for poultry meat and eggs. Efficiency of production systems and capacity to produce new poultry products rely on available genetic diversity among and across breeds.

Live conservation of poultry, both in situ and ex situ, is very expensive and carries the risk of mortality due to diseases, such as avian influenza. Cryopreservation of PGCs, which are egg and spermatozoa progenitors, provides an alternative way to preserve both male and female genetic material in poultry. Primordial germ cells in poultry can be harvested from the blastoderm, embryo's blood or from nine-day old gonads and preserved in liquid nitrogen like sperm, ovum and embryo in large ruminants. A generalized procedure illustrating the main points of the cryopreservation process is presented below (Tiersch 2011b):

- Sample collection and quality control

Collection of germplasm samples (fertile eggs in this case) must be performed by trained personnel who can identify fresh and fertile eggs. After collection, fresh samples are examined, cleaned and labelled. Only samples that meet specific quality criteria should be used. The biosanitary (disease) status of the flock must be noted.

- Sample packaging, sealing and labelling

The choice of egg packaging must be informed by several factors including reduced shock, efficient heat transfer, manual or automated processing, sealing and labelling and duration of storage.

- Cooling of samples

Cooling rate is a critical factor for cell survival and usually needs to be determined experimentally. Cooling can be achieved using programmable freezers, liquid nitrogen vapour, solid CO_2 (dry ice) or other methods where cooling rates are controllable.

- Sample storage in liquid nitrogen

Samples are commonly stored in liquid nitrogen (–196°C) or nitrogen vapour (–140 and –180°C) in specially designed storage equipment (e.g. vacuum flasks for cryogens). Theoretically, frozen samples could be stored at these temperatures indefinitely.

- Thawing for use

As with the cooling process, warming of frozen samples is critical for cell survival. Ideally, fast warming rates are preferred but not necessary. The warming process can usually be achieved by using a water bath at 40–60°C, a microwave (Ewert 1988) or infrared laser pulses (Jin and Mazur 2015; Daly et al. 2018). Samples are typically used for fertilization immediately after thawing.

Development of effective, robust and standardized cryopreservation protocols is essential to ensure cell survival and constitutes the core pathway for application of cryopreservation technology (Tiersch 2011). In addition to a robust cryopreservation pathway, procedures are required to ensure proper germplasm acquisition (including genetic background, sample numbers and quality, disease status), sample utilization of after thawing and product quality management (Hu et al. 2013; Hu et al. 2014). Furthermore, databases, sample inventory, protocols for daily maintenance of liquid nitrogen levels and marketing strategies must be developed for management of germplasm repositories that are meant for long term or commercial use (Torres and Tiersch 2018).

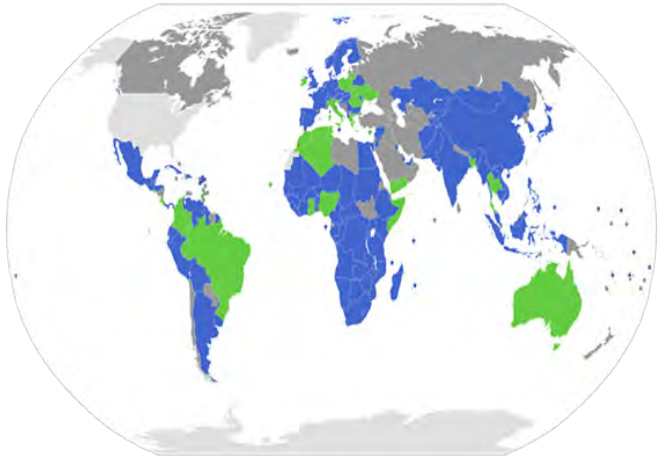
5. Fertile egg collection and processing

5.1 The legal compliance

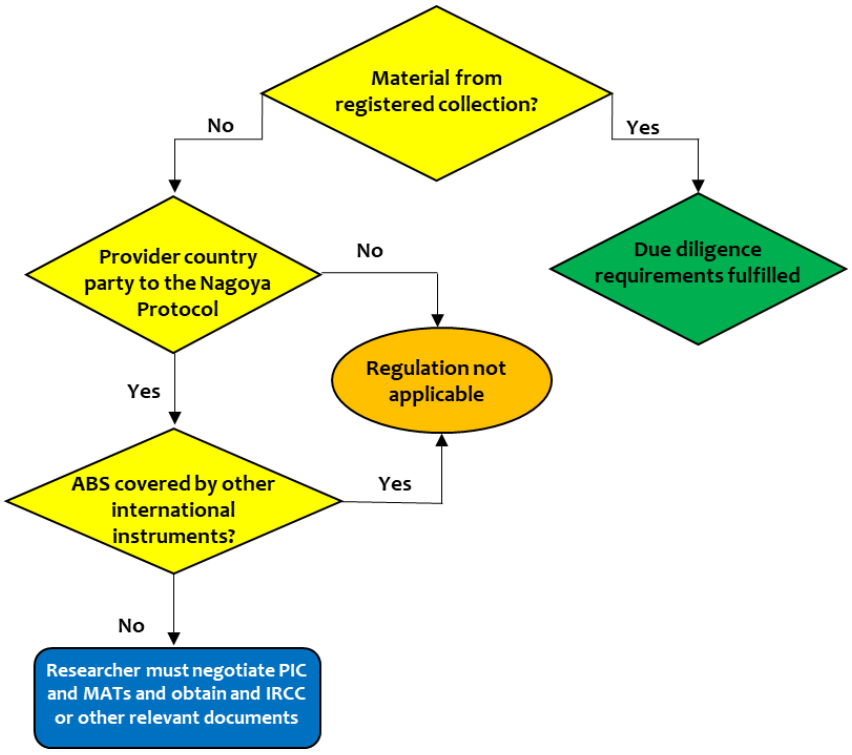
Compliance with the Access and Benefit-Sharing (ABS) framework of the Nagoya Protocol should be a priority when collecting all animal genetic resources (AnGR), including those from poultry. Highlighted below are some key considerations for compliance with the AnGR's Nagoya Protocol's ABS framework (Table 5):

Table 5. Key considerations for compliance with the ABS framework of the Nagoya Protocol for animal genetic resources

5.1.1 Initial planning stage	
Researcher receives funding for research Genetic resource (or its derivative) is identified by the researcher Researcher checks the ABS requirements and conditions of the provider country Researcher seeks guidance from the focal point and national competent authority on the prior informed consent (PIC) and mutually agreed terms (MAT) Researcher consults the Access and Benefit Sharing Clearing House (ABS-CH) and registers on the website (preferred method)	 
5.1.2 Is the Nagoya Protocol applicable?	
A – If the AnGR is obtained from a registered collection established in accordance with national or regional regulations, then requirements relating to due diligence are fulfilled. **B** – Check if the country is party to the Nagoya Protocol. **C** – Some countries have proposed implementing legal frameworks encompassing the ABS provisions of Nagoya, with the assumption that these will be accepted as an alternative to ABS.	



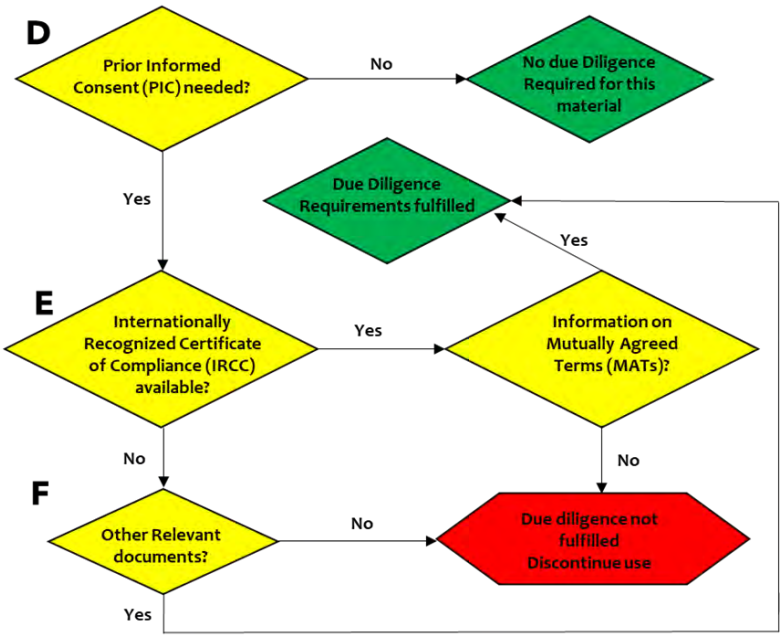
Parties
Only signed, but not ratified
Non signatory, but Biological Diversity Convention party
Non signatory, non-Biological Diversity Convention party



Apply for **PIC** as early as possible from:

- Competent national authorities • Relevant stakeholders • Different levels of government

5.1.3 Due Diligence



D – Parties may implement international agreements, which remove the need to obtain PIC (provided the agreements do not run counter to the objectives of the Protocol).

E – An internationally recognized certificate of compliance (IRCC) comprising information on PIC and MATs can be used as evidence that the AnGR has been accessed in accordance with the Protocol. This information can be used as part of the Seek, Keep and Transfer procedure and must be kept for more than 20 years post utilisation of the AnGR.

F – If an IRCC is not yet available, the researcher must Seek, Keep and Transfer other relevant documents related to the AnGR.

Due diligence paperwork

Ensure existing AnGR are sufficiently documented as having been accessed before the Nagoya Protocol effective date.

The Nagoya Protocol will not have retroactive effect. Hence, any AnGR accessed prior to the Nagoya Protocol will not be required to comply with the AU regulations.

Administrative preparations

Ensure administrative systems are in place so that any new AnGR are documented in compliance with the Nagoya Protocol.

Internal procedures

Carry out internal training to make staff aware of the Nagoya Protocol and the obligations of users of any AnGR. Ensure staff understand that legal possession of AnGR does not necessarily grant the holder the right to do any work on or with that resource.

Origins

Be aware of the origin of any AnGR to be used for research and development purposes.

What to do more of

Document the AnGR obtained

Be prepared for questions on the legal status of acquired AnGR

Consider your options in accessing AnGR

5.1.4 PIC and MAT negotiated and granted from the ABS CNA

Store all data documenting the PIC and MAT steps even if there is no due diligence obligation.

5.1.5 Start your research, respecting:

Local and national laws and regulations

Customs, traditions, values and customary practices of Indigenous People and Local Connunities (IPLCs)

Principles of conservation and sustainable use of biological resources

Cooperation with local researchers and institutions

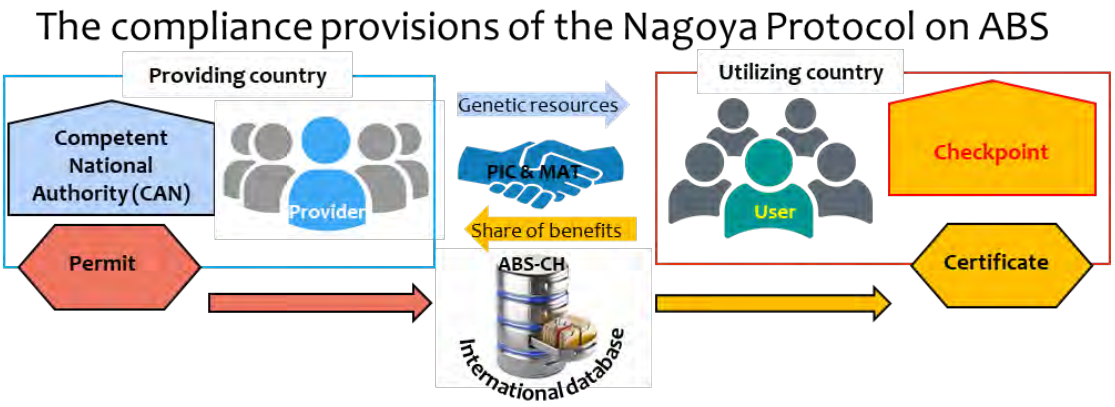
5.1.6 Share the results with stakeholders of the providing country

Share information with local people, communities and institutions

Make the research findings available to the ABS authority of the provider country

Provide your research partners with access to the findings

5.1.7 Seek and keep the internationally recognized certificate of compliance (IRCC) and transfer procedure

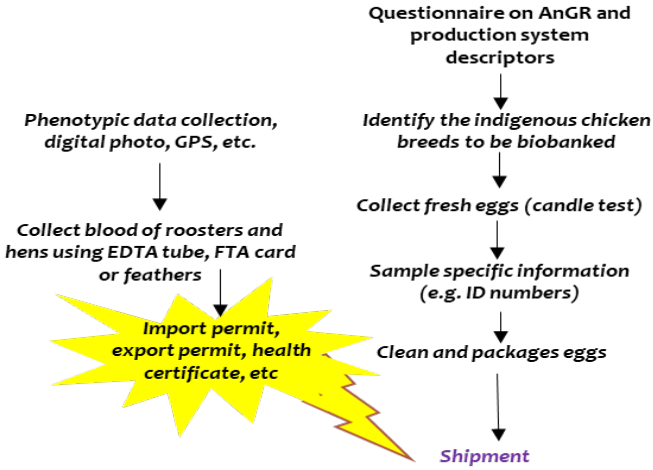


- PIC/MAT negotiated between user and competent national authority within the provider country using the ABS-CH as an internet-based platform
- Competent national authority issues a national permit to ABS-CH
- ABS-CH issues the international recognized certificate of compliance (IRCC) to applicant as evidence of compliance with the Protocol

5.2 Associated metadata

No genetic material should be granted access to the biorepository without proper documentation.

Table 6. Metadata associated with egg collections

i. Date and place of access. ii. Description of genetic resource utilized. iii. Direct source of AnGR/traditional knowledge and any subsequent users. iv. ABS agreements, access permits, MATs and any rights or obligations related to ABS.	

5.3 Processing, labelling, packaging and shipping

5.3.1 Processing of eggs

- Eggs should be processed at the production facility or collection site. They should be cleaned to remove excess moisture prior to packaging. Cleaning removes contaminants thus preventing egg spoilage. Collected eggs should be grouped according to similar characteristics, such as quality and weight. This requires the examination of internal quality factors e.g. condition of the egg white and yolk and air cell size through candling or external quality factors, such as shape, texture, cleanliness and soundness of the shell.

Table 7. Egg quality factors and their specifications

Quality specifications			
Quality factor	AA	A	B
Shell	Clean, unbroken and practically normal	Clean, unbroken and practically normal	Clean to slightly stained*, unbroken and abnormal
Air cell	3 mm or less in depth, unlimited movement and free or bubbly	6 mm or less in depth, unlimited movement and free or bubbly	Over 6 mm in depth, unlimited movement and free or bubbly
Egg white	Clear and firm	Clear and reasonably firm	Small blood and meat spots present**
Yolk	Outline slightly defined, practically free from defects.	Outline fairly well defined, practically free from defects	Outline plainly visible, enlarged and flattened with clearly visible germ development but no blood or other serious defects
For eggs with dirty or broken shells, the standards of quality provide two additional qualities:			
Dirty		Other quality Check	
Unbroken with adherent dirt or foreign material. Prominent stains or moderately stained areas more than B quality		Broken or cracked shell but membranes intact and not leaking ***	
* Moderately stained areas permitted (0.75 mm of surface if localized or 1.5 mm if scattered) ** Small (aggregating not more than 3 mm in diameter) *** Egg has broken or cracked shell membranes and contents leaking or free to leak			

Source: United States Department of Agriculture

The size of eggs is determined by weight. Accordingly, there are six different weight categories as shown in Table 8.

Table 8. Size categories of eggs based on weight

Weight Category	Weight (grams)
Peewee	35-42
Small	42-49
Medium	49-56
Large	56-65
Extra large	65-70
Jumbo	70 and above

5.3.2 Labelling

The label contains important facts/information on the eggs, such as size, weight and quality/grade description. Labels may also indicate the farm of origin (geographical location), date when the eggs were laid, information on the laying hens and breeding flock, where possible.

5.3.3 Packaging

Despite its relative strength, an egg is an extremely fragile product and even with the best handling methods, serious losses can result from shell damage. Packaging is an important component in delivering quality eggs to the laboratory. It embraces both the art of preparing products for storage and transport. Packaging protects eggs from microorganisms, natural predators, moisture loss, tainting, deterioration from suboptimal temperatures and possible crushing during handling, storage and transportation.

Proper handling and storage also help to control moisture loss from eggs. Eggs also need to breathe, hence the packaging material used must let in air. The material used must be clean and odourless to prevent possible contamination and tainting. Authentic egg packaging materials can be reused. Packaging must be made to withstand handling, storage and diverse transportation methods. It must also protect the eggs against temperatures and humidity that may cause deterioration. Many factors must be taken into consideration when packaging eggs. These include quality maintenance, storage facilities, transport type, travel distances, climatic conditions, time etc.

As described in Table 9 below, there are many different types of egg packages, which vary both in design and packaging material used.

Table 9. Egg packaging types

Type	Description of packaging	Advantages
1	Eggs packed with clean and odourless rice husks, wheat chaff or chopped straw in a firm walled basket, crate or simple basket with cushioning material	Reduces risk of shell damage
2	Filler tray placed in boxes or cases Filler trays are made of wood pulp moulded to accommodate the eggs Constructed so that they can be stacked and placed in boxes ready for transport	Filler trays offer a convenient method for counting the eggs in each box, without having to count every single egg
3	Smaller packaging made of paperboard or moulded wood pulp/plastic or paperboard cases covered with plastic film, polystyrene etc.	Polystyrene provides better cushioning and protection against odours and moisture in addition to resistance to fungus and mould growth

5.3.4 Storage of eggs

For the successful storage of eggs, the following conditions must be met:

- The eggs must be clean; they must not be washed or be wet.
- Packaging material should be clean and odourless.
- Loss of water due to evaporation should be minimized.
- The storage room must be free from tainting products or materials and should be cleaned regularly with odourless detergent sanitizers.
- The storage room must be kept at a constant temperature and appropriate humidity.
- There should be air circulation in the storage room.
- Eggs should be able to ‘breathe’ while in storage.
- As far as possible, interior quality should be monitored; there should be a good proportion of thick egg white and the yolk should stand up well.

5.3.5 Cold storage of eggs

In the tropics, eggs can deteriorate very quickly unless they are stored at low temperatures. The ideal temperature for storage in such climates is 13°C or lower (usually between 11 and 15°C). The most important parameter is how long to store eggs. 7 to 10 days at 14C

The most important factors for successful cold storage are:

- The selection and packaging of eggs
- The equipment and preparation of the cold store
- Proper temperature, humidity and air circulation
- Periodic testing for quality
- The gradual adjustment of eggs to higher temperatures when removed from storage

5.3.6 The selection and packaging of eggs for storage

Eggs for storage must have a sound, clean shell with satisfactory internal quality parameters. Eggs destined for incubation should not be stored for more than seven days, as the hatchability starts to decrease thereafter. The eggs should be kept cool during storage. When packaging material is reused, it is extremely important that it is clean, taint-free and allows the eggs to 'breathe'. It should also be sturdy if the cases are to be stacked.

For storage of large quantities of incubation eggs, the storage room should have washable concrete floors, walls and ceilings. Wooden buildings have been found to be satisfactory, provided they do not impart foreign odours or flavours to the eggs. The room should be scrubbed thoroughly with hot water and soap or an odourless detergent sanitizer prior to use. A final rinse with a hypochlorite solution helps greatly in deodorizing the storeroom. A liberal application of freshly slaked lime to unpainted plaster surfaces also helps. After cleaning, the storage room should be aired and dried out thoroughly before the doors are closed and the refrigeration turned on. It is best to allow the temperature and humidity to stabilize for several days before introducing the eggs.

Careful and accurate control of the air conditioning is essential because eggs will freeze at a temperature of -2.5°C. The room should be well constructed and insulated. The refrigeration should be capable of maintaining adequate uniform temperature in all areas. The egg cases should be separated by wood strips and kept well away from the walls to avoid air circulation obstruction. Aisles left for the convenience of handling specific egg cases also help with air circulation. Periodic ventilation of the storage room is advisable to promote air exchange.

At cold storage temperatures of about 10°C, the relative humidity should be maintained at 75-80%. In such instances, egg weight loss should not exceed 0.5% on average. During the early stages of storage when the packaging material is absorbing moisture at a high rate, the floors should be sprinkled with clean water several times a day. If forced-air circulation is feasible, a controlled temperature water-spray air washer may be used. If the humidity becomes excessive, part of the air can be cycled through a unit containing calcium chloride. Where eggs have been oiled, less attention can be paid to the humidity level.

5.3.7 The gradual adjustment of eggs to higher temperatures

Care must be taken when removing eggs from cold storage to avoid the condensation of moisture on shells. This is minimized by raising the temperature slowly or by moving the eggs through rooms with intermediate temperatures. If condensation occurs, the eggs should be held under conditions that allow the moisture to evaporate within a day.

The layout for the packaging and storage facility is of great importance for efficient and effective management. The various rooms should be kept clean, well ventilated and where necessary, refrigeration must be provided. All personnel working in the facility should wear clean outer garments, use caps or head bands and wash their hands when handling eggs and equipment. All equipment used should be clean.

5.3.8 Shipping eggs

For the successful transportation of eggs, three essential requirements must be met:

a. The containers and packaging materials must be such that the eggs are well protected against mechanical damage.

b. Care should be taken at all stages of handling and transportation. Staff handling eggs should do so carefully.

c. The eggs must always be protected from extreme temperatures as well as contamination that may affect the embryos.

The permissible range of temperatures during transportation depends on local climatic conditions and the duration of the journey. Table 10 shows recommended temperatures during loading and transportation of eggs.

Table 10. Recommended temperatures during loading and transportation of eggs

	Transportation over 2 to 3 days	Transportation over 5 to 6 days
Maximum temperature on loading (°C)	+6	+3
Recommended temperature during transportation (°C)	-1 to +3	-1 to +1
Acceptable temperature during transportation (°C)	1 to +6	1 to +3

Care is needed to avoid excessive shaking, especially where roads are bad. Egg containers should be stacked tightly and tied down securely to minimize movement. Covers should be used to protect eggs from the sun, rain and extreme cold, where applicable. Where bicycles are used, a device such as a special carrier suspended on springs may be helpful. A basic prerequisite for all long-distance transportation is that arrangements must be made for proper reception, handling and storage at the end of the journey to avoid substantial quality deterioration.

Requirements for the successful operation of refrigerated transport equipment are rigid especially with regards to the following factors:

- Efficiency and durability of insulation
- Adequacy and reliability of the cooling mechanism and
- Adequate circulation of air within the vehicle or container to minimize temperature variations.

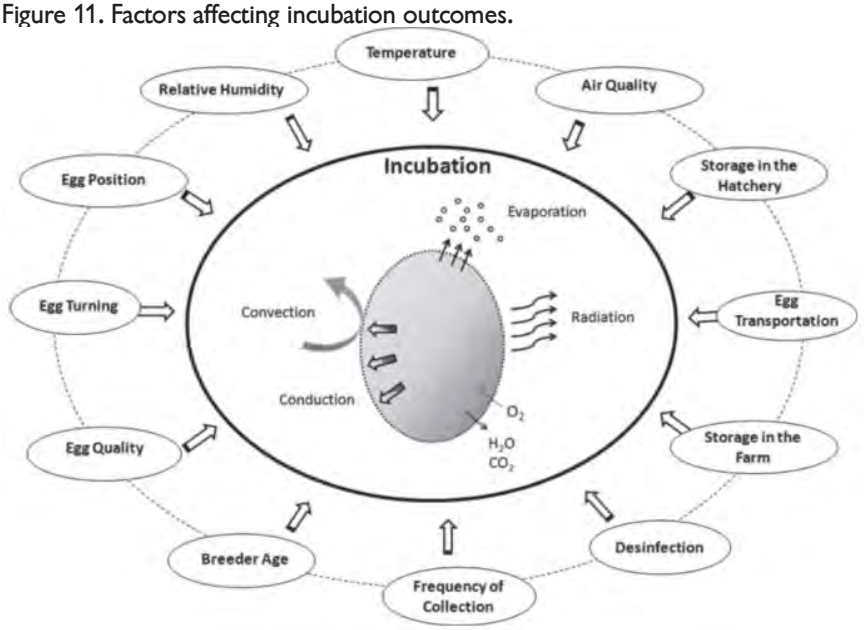
6. Egg incubation process

6.1 Hatchery operation and sanitation

- The incubation and hatchery room (dirty lab) should be separate from the cell harvesting and injection room (clean lab).
- The timing of eggs setting in the incubator should carefully take the time and type of experiment, as well as the capacity of the operator or the team to work with that number of eggs within the period of egg development stage required.
- Avoid using substandard eggs (weighing less than 32 grams, cracked or dirty).

Several factors (Figure 11) can affect incubation outcomes.

Figure 11. Factors affecting incubation outcomes.



Source: Boleli et al., 2016

Physical exchanges between eggs and the environment during incubation (heat transfer, water loss and gaseous exchanges) depend on the egg characteristics (size, composition, form and eggshell thickness, porosity, heat and water vapour conductance), embryo metabolism rate, physical incubation conditions and the pre-incubation conditions.

The four main requirements for incubation of good quality fertile eggs are:

- Correct and even temperature monitored by a thermometer or thermocouple

- Correct humidity controlled by ventilation rate and water application
- Correct oxygen and carbon dioxide concentrations controlled by ventilation
- Turning (approximately 90°) of fertile eggs several times per day by manual or automatic means

These parameters can be easily achieved and maintained if the incubator's operating instructions are carefully adhered to.

6.2 Incubation temperature and relative humidity

The temperature requirements for incubation are described in Table 11 below. Most incubators have a temperature variation of 0.2-0.4°C for effective incubation and a high hatchability rate.

Embryos have low tolerance to temperature variations of more than 1°C above or below the recommended level. Temperatures outside the recommended range will result in significant embryonic mortality. Embryos are particularly susceptible to temperature variation in the early and late phases of incubation.

Table 11. Incubation period and recommended temperature and humidity ranges for various poultry species

	Chicken	Turkey	Duck	Muscovy Duck	Goose	Pheasant	Guinea Fowl	Quail
Incubation period (days)	21	28	28	35	28	23-28	28	23-24
Incubation temperature (°C)	37.6	37.4	37.5	37.5	37.4	37.6	37.6	37.6
Wet bulb temperature (°C)	29.4–30.5	28.3–29.4	28.8–30	28.8–30	30–31.1	30–31.1	28.3–29.4	28.8–30
Relative humidity (%)	56–62	51–56	53–60	53–60	60–65	60–65	51–56	53–60
Number of turns per day	18	25	25	31	25	21	25	21
Incubation temperature (°C) (last 3 days)	37.4	37.2	37.3	37.3	37.2	37.4	37.4	37.4
Wet bulb temperature (°C) (last 3 days)	32.2–34.4	32.2–34.4	32.2–34.4	32.2–34.4	32.2–34.4	33.3–35	32.2–34.4	32.2–34.4
Relative humidity (%) (last 3 days)	70–83	70–83	70–83	70–83	70–83	76–90	70–83	70–83

Source: Poultry Hub Australia, 2021

For still air incubators, add approximately 1°C to the operating temperatures recommended in Table 11. This is because the thermometer in still air incubators is normally located at the top of the incubator and there is a marked temperature gradient from the top of the incubator to the bottom.

A consistent relative humidity is difficult to achieve during incubation and can only be maintained by ventilation rate, using adjustable ventilation apertures and by using surface water or water sprays during incubation. The tolerance of the embryo to variation in humidity is greater than its tolerance for temperature variations. However, there are negative consequences observed when humidity falls below 40% or exceeds 90%. Good hatchability is achieved when relative humidity is maintained at approximately 50–65% until the last three days of incubation, at which point it should be increased to 70–90%.

6.3 Ventilation and carbon dioxide/oxygen concentration

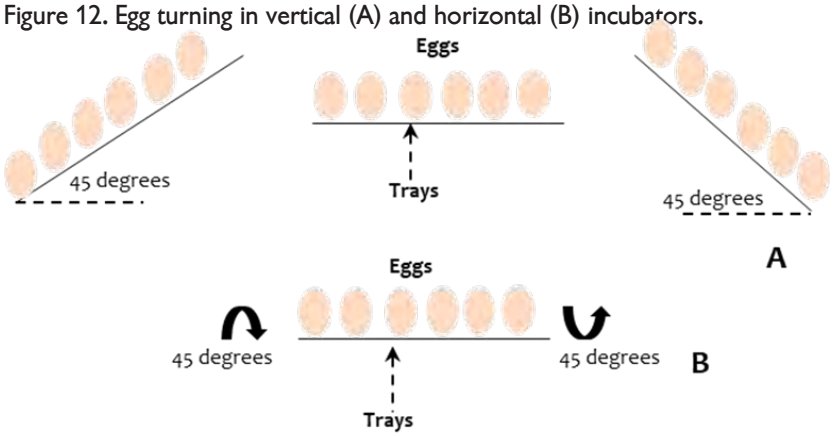
Embryonic growth is optimized at a carbon dioxide concentration of 0.4%. Conversely embryonic growth is depressed and mortality increased when carbon dioxide concentrations exceed 1%. The normal atmosphere contains 21% oxygen and 0.04% carbon dioxide. The hatched chick is most susceptible to oxygen deviation (compared to the pipped chick and

the embryo in the intact egg), which implies that ventilation rate and carbon dioxide concentration are most critical in the late phase of incubation.

6.4 Egg turning /rotation

Egg rotation or turning is required to ensure that the embryo developing on the yolk does not adhere to the shell membrane. This phenomenon of adherence to the shell membrane commonly occurs during storage and early incubation (generally the first week) of fertile eggs. The turning process allows the embryo to revolve and slide in the inner white and provides it with access to additional nutrients for embryonic development. Egg turning should be done three to six times per day and an uneven number of rotations are better so that the eggs are not in the same position for long periods. Most incubators automatically rotate the eggs by approximately 90°.

Figure 12. Egg turning in vertical (A) and horizontal (B) incubators.



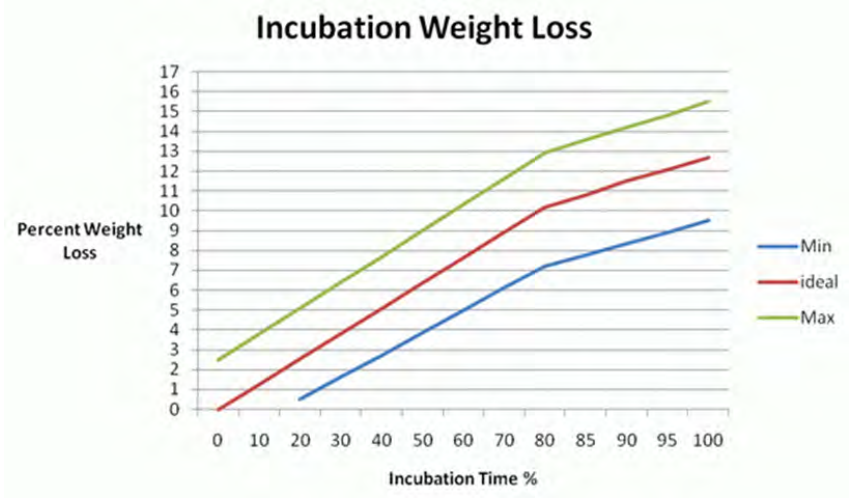
Adapted from Boleli et al., (2016)

6.5 Candling of incubated eggs

Eggs should be examined at appropriate times using a candling light to assess the embryo for blood vessel development ('spider web-like') and a dark spot. Infertile eggs are obviously clear with no evidence of blood and early embryonic death is noted by the presence of a blood ring surrounding the yolk. Infertile eggs and dead embryos are removed at this stage. Candling can also be undertaken at 18 days of incubation, where the embryo is clearly visible with a distinct dividing line between the embryo and the air cell.

Eggs with a growing embryo will progressively dry out throughout incubation. This results in an overall weight loss of the egg. This progressive weight loss can be objectively monitored to ensure incubation success. Figure 13 below is a guide for monitoring egg weight loss during incubation. The ideal total weight loss is 13%.

Figure 13. Ideal, minimum and maximum percentage weight loss of eggs during incubation.



Source: Poultry Hub Australia, 2021

When all measures are properly taken, the operator will have healthy and active chicks by Day 21. Failing to follow the normal procedures will result in various incubation and hatching problems. Table 12 presents some of the incubation problems and possible causes, while Figure 14 illustrates the embryo development process.

Table 12. Possible causes of hatching problems

Observation	Possible cause(s)
Eggs exploding	Dirty eggs Improperly cleaned eggs Dirty incubator
No embryonic development	Infertile egg Rough handling of eggs Incubation temperature too high or too low Eggs stored too long prior to incubation Eggs stored improperly Breeders stressed or too many hens per rooster Old or unhealthy hens or males Inbreeding or disease
Blood ring (early embryonic death)	Old eggs Incubation temperature too high or too low Electric power failure Eggs not turned Inbreeding Infection Poor nutrition of breeders
Air cell too small	Humidity too high
Air cell too large	Humidity too low
Chicks hatch early, dry chicks, bloody navels, chicks too small	Small eggs Temperature too high Humidity too low
Chicks hatch late	Large eggs Old eggs Temperature too low Humidity too high
Chicks dead after pipping	Eggs not turned during the first two weeks Thin-shelled eggs Temperature too low or too high during incubation Humidity too low or too high during incubation Infection and/or disease
Unhealed navel (mushy chicks)	Temperature too low during incubation Wide temperature variation in incubator Humidity too high during incubation Poor ventilation

Malformed legs and toes	Improper temperature during incubation Improper humidity during incubation Legs may be also injured during hatching or handling of chicks
Weak chicks	Temperature too high or low Old eggs Poor ventilation
Gasping chicks	Disease: Bronchitis or Newcastle disease
Malpositions	Temperature too high or low Inadequate turning Large (rounded) end of egg not up when set Old or poorly handled eggs Poor breeder nutrition

Figure 14. Chick embryo development.

Table 13. Hamburger and Hamilton (1951) (HH) chronological stages of chick embryo development

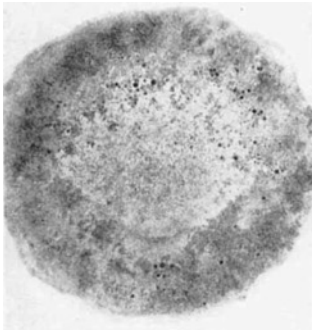
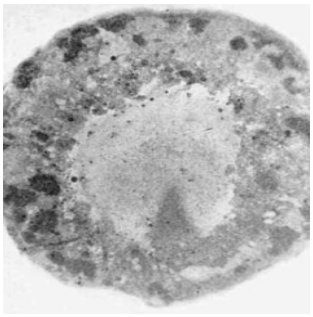
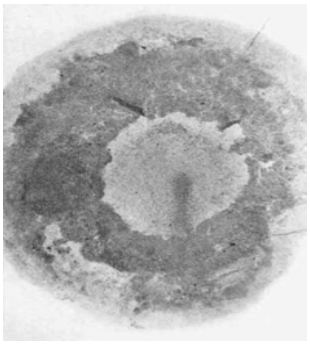
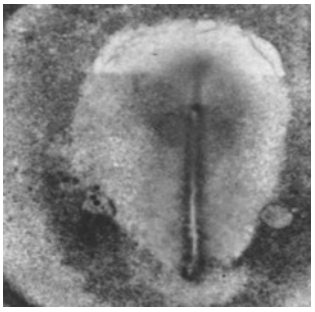
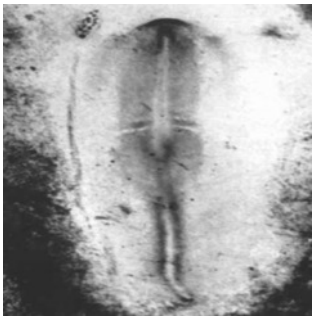
Stages	Incubation time/days		Main embryonic event
HH 1	First few hours of incubation		Pre-streak Embryonic shield
HH 2	6–7 hours		Initial streak Pre-gastrulation
HH 3	12–13 hours		Intermediate streak Onset of gastrulation
HH 4–5	18–22 hours		Definitive streak to head process Gastrulation The notochord is visible as a rod of condensed lateral plate mesoderm
HH 6–7	23–26 hours		Head fold Three somites Neurulation

Table 13. Hamburger and Hamilton (1951) (HH) chronological stages of chick embryo development

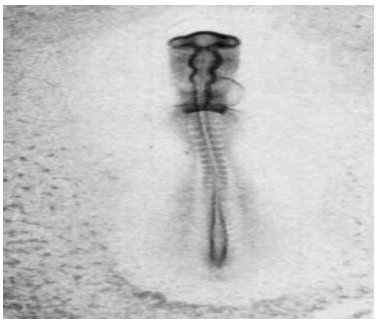
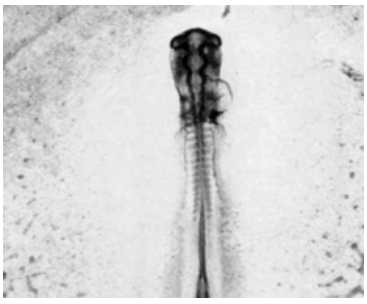
Stages	Incubation time/days		Main embryonic event
HH 8	26–29 hours		4–6 somites Neural folds meet at level of midbrain Blood islands are present in posterior half of blastoderm
HH 9	29–33 hours		7–9 somites Primary optic vesicles are present Paired primordia of heart begin to fuse
HH 10–11	33–45 hours		10–15 somites Three primary brain-vesicles are clearly visible Optic vesicles not constricted at bases Slight cranial flexure Five neuromeres of hindbrain are distinct Anterior neuropore is closing Optic vesicles are constricted at bases Heart bent to right
HH 12-13-	45–49 hours		16–19 somites Head is turning onto left side and is slightly S-shaped Anterior neuropore closed Telencephalon indicated Primary optic vesicles and optic stalk well established Auditory pit is deep, but wide open Heart head fold of amnion covers the entire forebrain

Table 13. Hamburger and Hamilton (1951) (HH) chronological stages of chick embryo development

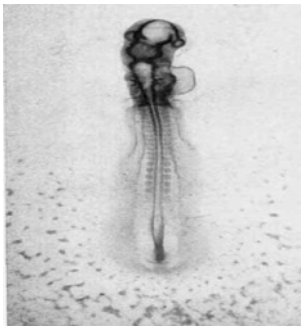
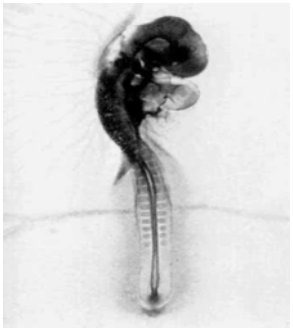
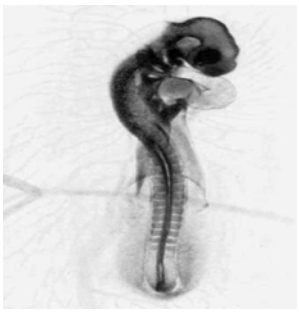
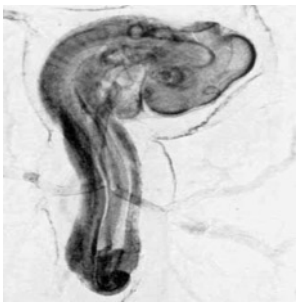
Stages	Incubation time/days		Main embryonic event
HH 13+	50–52 hours		20–21 somites Head is partly to fully turned to the left Cranial and cervical flexures make broad curves Distinct enlargement of telencephalon Slight narrowing of opening to deep auditory pit No indication of hypophysis Atrio-ventricular canal indicated by constriction Head-fold of amnion covers forebrain, midbrain and anterior part of hindbrain
HH 14–15	50–55 hours		22–27 somites Flexures and iotatiow Cranial flexure: axes of forebrain and hindbrain form about a right angle
HH 16	51–56 hours (2.1 days)		26–28 somites Lateral body folds extend to somites 17-20, between levels of wings and legs Wing is lifted off blastoderm by infolding of lateral body fold. It is represented by a thickened ridge Primordium of leg is still flat, represented by a condensation of mesoderm
HH 17–18	52–69 hours (2.5 days)		29–36 somites Lateral body folds extend around the entire circumference of the body Both wing and leg buds are lifted off blastoderm by infolding of the body folds Both are distinct swellings of approximately equal size Limb buds enlarged; stage 18 leg buds slightly larger than wing buds The rotation extends now to the posterior part of the body hence the leg buds are no longer in the horizontal plane The tail bud is turned to the right, at about an angle of 90° to the axis of the posterior trunk

Table 13. Hamburger and Hamilton (1951) (HH) chronological stages of chick embryo development

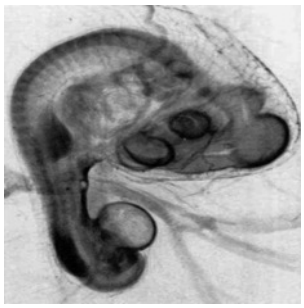
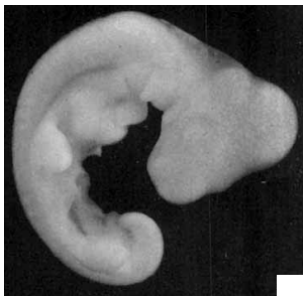
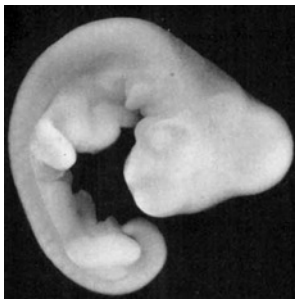
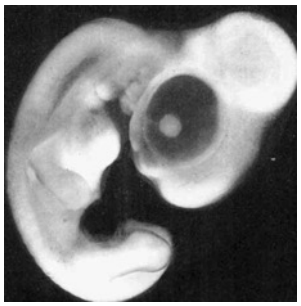
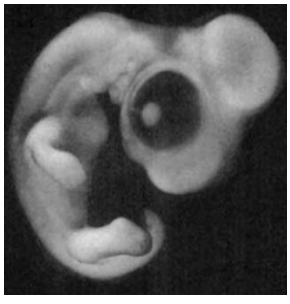
Stages	Incubation time/days		Main embryonic event
HH 19-20	68–72 hours (3.0 days)		37–43 somites Limb buds are enlarged and symmetrical Leg buds slightly larger and bulkier than wing buds Tail bud curved, its tip pointing forward Eyes not yet pigmented at stage 19 Eyes have a faint grayish hue at stage 20
HH 21-23	3.5–4 days		43–44 somites At stage 21, limbs are enlarged Both wing and leg buds are slightly asymmetrical; their proximo-distal axes are directed caudal The posterior contours of wing and leg buds are steeper than the anterior contours, they meet the baseline at an angle of approximately 90°
HH 24	4.0 days		All somites formed Wing and leg buds distinctly longer than wide Digital plate in wing not yet demarcated Toe plate in leg bud distinct Toes not yet demarcated
HH 25-26	4.5–5 days		Elbow and knee joints distinct (in dorsal or ventral view) Digital plate in wing distinct, but no demarcation of digits Indication of faint grooves demarcating the third toe on leg At stage 26, indication of faint groove between second and third digit Demarcation of the first three toes distinct
HH 27	5.0 days		Contour of digital plate angular in region of first digit Grooves between first, second and third digits indicated Grooves between toes are distinct on outer and inner surfaces of toe-plate First toe projects over the tibia part at an obtuse angle Tip of third toe not yet pointed

Table 13. Hamburger and Hamilton (1951) (HH) chronological stages of chick embryo development

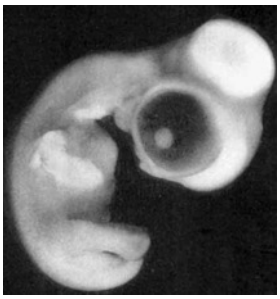
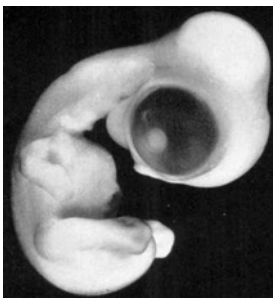
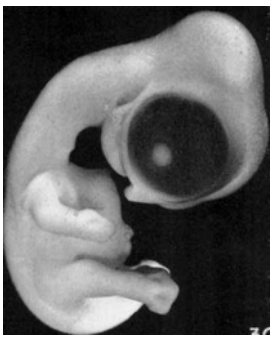
Stages	Incubation time/days		Main embryonic event
HH 28	5.5 days		Second digit and third toe longer than others, which gives the digital and toe-plates a pointed contour Three digits and four toes distinct No indication of fifth toe A distinct outgrowth of beak is visible in profile
HH 29	6.0 days		Wing bent in elbow Second digit distinctly longer than the others Shallow grooves between first, second and third digits Second to fourth toes stand out as ridges separated by distinct grooves and indications of webs between them Distal contours are straight lines, occasionally with indication of convexity Rudiment of fifth toe visible The beat is more prominent, but no egg tooth visible yet
HH 30	6.5 days		The three major segments of wing and leg are clearly demarcated Wing bent in elbow joint, while the leg is bent in knee joint Distinct grooves between first and second digits Contours of webs between first two digits and between all toes are slightly curved concave lines The mandibular process approaches the beak, but the gap between the two is still conspicuous Lengthening of neck between 'collar' and mandible is very conspicuous. 'Collar' begins to flatten Two dorsal rows to either side of the spinal cord at the brachial level Three rows at the level of the legs; they are rather indistinct at thoracic level and none on the thigh Egg tooth distinct, slightly protruding Beak more pronounced than in previous stage

Table 13. Hamburger and Hamilton (1951) (HH) chronological stages of chick embryo development

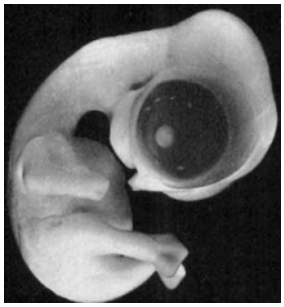
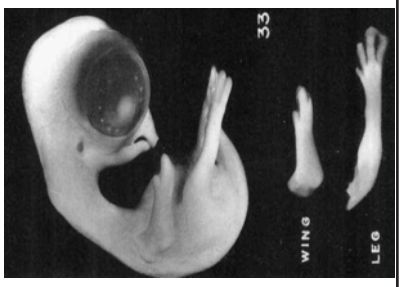
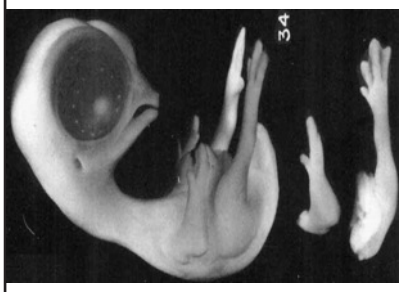
Stages	Incubation time/days		Main embryonic event
HH 31	7.0 days		Indication of a web between first and second digits Rudiment of fifth toe still distinct The gap between mandible and beak has narrowed to a small notch 'Collar' inconspicuous or absent Approximately seven rows at lumbo-sacral level Distinct feather papillae on thigh One indistinct row on each lateral edge of the tail
HH 32-33	7.5–8 days		All digits and four toes have lengthened conspicuously Rudiment of fifth toe has disappeared Webs between digits and toes are thin and their contours are concave Differences in size of individual digits and toes become conspicuous Anterior tip of mandible has reached the beak 'collar' has disappeared or is faintly recognizable Eleven rows or more on dorsal surface at level of the legs One row on tail distinct and second row indistinct Scapular and flight feather germs barely perceptible at optimal illumination or absent For the tail, three rows distinct, the middle row considerably larger than the others Thirteen scleral papillae, forming an almost complete circle, with gap for one missing papilla at a ventral point near the middle of the jaw
HH 34	8.0 days		Differential growth of second digit and third toe conspicuous Contours of webs between digits and toes are concave and arched Lengthening of mandible and of neck continues feather-germs on scapula, on ventral side of neck, on pro-coracoid and posterior (flight) edge of wing are visible under good illumination Feather germs next to dorsal midline, particularly at lumbo-sacral level extend slightly over surface when viewed in profile Feather germs on thigh protrude conspicuously. One row on inner side of each eye and none around umbilical cord Nictitating membrane extends halfway between outer rim of eye (eyelid) and scleral papillae

Table 13. Hamburger and Hamilton (1951) (HH) chronological stages of chick embryo development

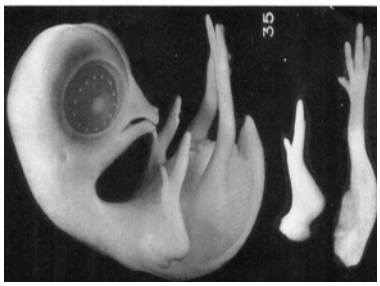
Stages	Incubation time/days		Main embryonic event
HH 35	8–9 days		Webs between digits and toes become inconspicuous. A transitory protuberance on the ulnar side of the second digit is probably a remnant of the web Phalanges in toes are distinct Lengthening of beak continues Feather-germs are all more conspicuous Mid-dorsal line stands out distinctly in profile view At least four rows on inner side of each eye. New appearance of feather germs near mid-ventral line, close to sternum and extending to both sides of umbilical cord Nictitating membrane has grown conspicuously and approaches the outer scleral papillae Eyelids (external to nictitating membrane) have extended towards the beak and have begun to overgrow the eyeball The circumference of the eyelids has become ellipsoidal

Table 13. Hamburger and Hamilton (1951) (HH) chronological stages of chick embryo development

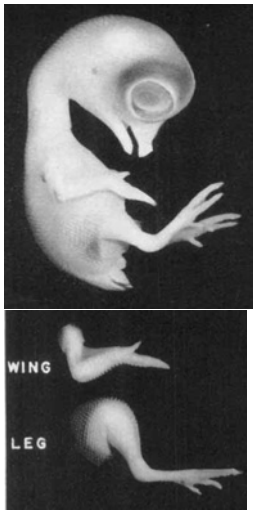
Stages	Incubation time/days		Main embryonic event
HH 36	10 days		Distal segments of both wing and leg are proportionately much longer Length of third toe, from its tip to the middle of its metatarsal joint = 5.4 ± 0.3 mm. Tapering primordia of claws are just visible on termini of the toes and on digit one of the wings Protuberance on posterior side of digit two of wings is missing Primordium of the comb appears as a prominent ridge with slightly serrated edge along the dorsal mid- line of the beak A horizontal ('labial') groove is clearly visible at the tip of the upper jaw, but is barely indicated on the tip of the mandible Nostril has narrowed to a slit. Length of beak from anterior angle of nostril to tip of bill = 2.5 mm Flight feathers are conspicuous and coverts are just visible in web of wing Feather germs now cover the tibio-fibular portion of the leg At least 9-10 rows of feather germs between each upper eyelid and the dorsal midline Sternal tracts prominent, with 34 rows on each side of ventral midline when counted in anterior part of sternum, merging into many rows around the umbilicus Nictitating membrane covers anterior most scleral papillae and approaches cornea Lower lid has grown upward to level of cornea Circumference of lids is a narrowing ellipse with its ventral edge flattened

Table 13. Hamburger and Hamilton (1951) (HH) chronological stages of chick embryo development

Stages	Incubation time/days		Main embryonic event
HH 38	12 days		Primordia of scales are marked off over entire surface of leg and ridges have not yet grown out to overlap surface Tips of toes show a ventral center of cornification as well as the more extensive dorsal one Main plantar pad is ridged when seen in profile. Length of third toe = 8.4 ± 0.3 mm. Labial groove marked off by a deep furrow at the end of each jaw Length of beak from anterior angle of nostril to tip of bill = 3.1 mm Coverts of web of wing are becoming conical. External auditory meatus is surrounded by feather germs Sternum is covered with feather germs except along midline Upper eyelid is covered with newly formed feather germs and the lower lid is naked except for two to three rows at its edge Lower lid covers tw0 thirds to three fourths of cornea. Opening between lids is much reduced
HH 40 -44	14 days		Length of beak from anterior edge of nostril to tip of bill = 4.0 mm The main channel of the auditory meatus is not visible in strictly lateral view of its external chamber Length of third toe = 12.7 ± 0.5 mm. Scales overlapping on inferior as well as superior surfaces of leg Dorsal and ventral loci of cornification extend to base of exposed portion of toenail Entire plantar surface of phalanges is covered with well-developed papillae Beak's length from anterior angle of nostril to tip of upper bill = 5.7 mm The translucent peridermal covering of the beak is starting to peel off proximally Length of third toe = 20.4 ± 0.8 mm.
HH 46	20–21 days		Newly hatched chick

Pictures references:Hamburger and Hamilton (1951)

* Hutterstock, Royalty-free stock photo ID: 84408997

7. Collection of embryonic tissues containing PGCs from target lines or breeds

7.1 Collection of blood for PGC cultivation

This refers to the collection of PGCs from fresh embryonic blood by aspiration. The objective is to isolate avian germ cells from early embryonic blood of fertile avian eggs and propagate them in culture.

7.1.1 Equipment/Reagents

1. Humidified 37°C incubator
2. Category 2 tissue culture suite
3. Egg incubator
4. Flow bench hood
5. Germ cell culture medium (DMEM containing chicken serum and recombinant growth factors)
6. Poultry fertilized eggs
7. Scissors/ tweezers sterilized in 70% ethanol
8. 70% ethanol
9. Pulled microcapillary tubes
10. Sigma aspirating tube (Sigma A5177) consisting of plastic mouthpiece, latex tubing (38.1 cm) and silicone rubber nosepiece
11. Eppendorf tubes
12. Blue roll petri dishes
13. Yellow waste bag
14. Pipette and 10 µl tips

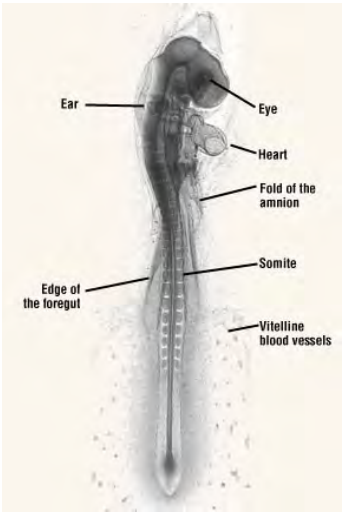
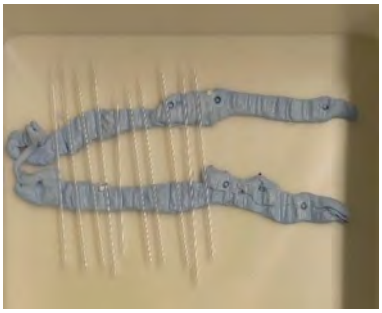
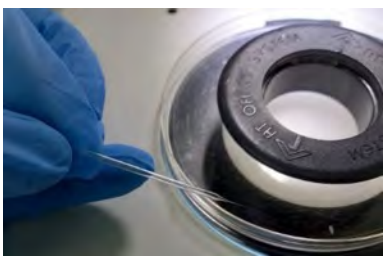
7.1.2 Safety

- Chicken embryos do not contain infectious material, so they are not a biohazard. However, appropriate care must be taken and appropriate personal protection (e.g. blue nitrile gloves and lab coat) must be worn during the

procedure.

- Aspirator must be rinsed with tape water, sprayed with 70% ethanol, ad air dried before use on kimwipes or blue towel before use. The same protocol must be used when finished to eliminate the possibility of bacterial egg product contamination
- Glass microcapillaries must be disposed in a sharps bin for incineration immediately after use. Care must be taken not to leave used microcapillaries on bench tops.
- Egg waste is discarded into yellow bags for incineration.
- Care must be taken not to put the mouthpiece in contact with any lab surfaces. Even the blue towel could present problems after a short time on a laboratory bench top setting. It is best practice to drape the apparatus over the microscope headpiece so neither end touches any lab surface.
- Blood aspiration should only be done in the chick embryology room.
- Care must be taken not to touch the mouthpiece after handling egg contents with gloves, which should be changed frequently. Raw egg products should be treated with care.

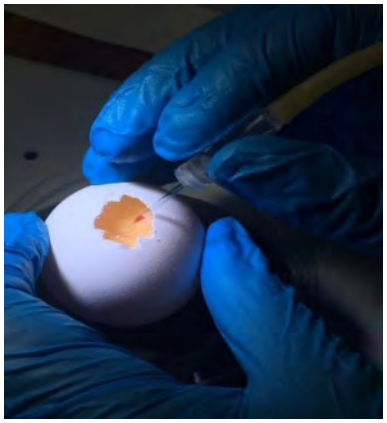
7.1.3 Procedure

7.1.3.1 Eggs are placed in the incubator early on Day 1 (e.g. Tuesday morning, 9am) with or without rocking. They are removed and left at room temperature two days later (Thursday) at 5pm when they are 56 hours old. Embryos can be sampled then or they are left overnight at room temperature and re-incubated They are incubated early on Friday to warm them up for a few hours. At this point, embryos should be around stage 16 (see Table 13) but not older than stage 17. http://chickscope.beckman.illinois.edu/explore/embryology/day02/33hour.html	
7.1.3.2 Under the microscope, break the end of a capillary using ethanol sterilised forceps then insert the large open end into an aspiration tube. Drape aspirator apparatus over the microscope making sure that both ends do not come into contact with laboratory surfaces. Maintain this position throughout the procedure.	 

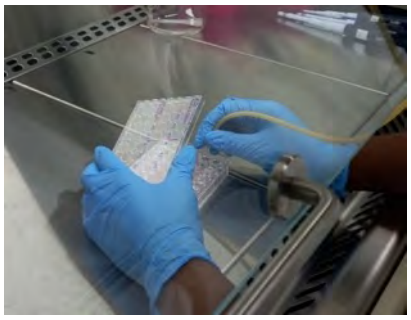
7.1.3.3 Take one egg from incubator. Lightly spray egg with ethanol and make a small window using a forcep. The heart of the embryo should be visibly beating.

If the egg is in right stage, such as HH15-16, the rest of eggs should be taken out of incubator and cool on a bench at room temperature.

7.1.3.4 Blow bubble onto surface of egg using capillary to demonstrate that the needle is open. Insert capillary into the dorsal aorta at a shallow angle. Aspirate 1-2 µl of embryonic blood into capillary.

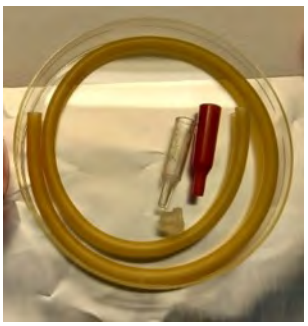


7.1.3.5 Go to the tissue culture hood in the laboratory. Drape aspirator apparatus over microscope so both ends do not touch laboratory surfaces while the plate is positioned. Blow blood sample into one or two wells (containing 300 µl medium each) of a 48-well tissue culture plate. Repeat for up to 20 embryos.



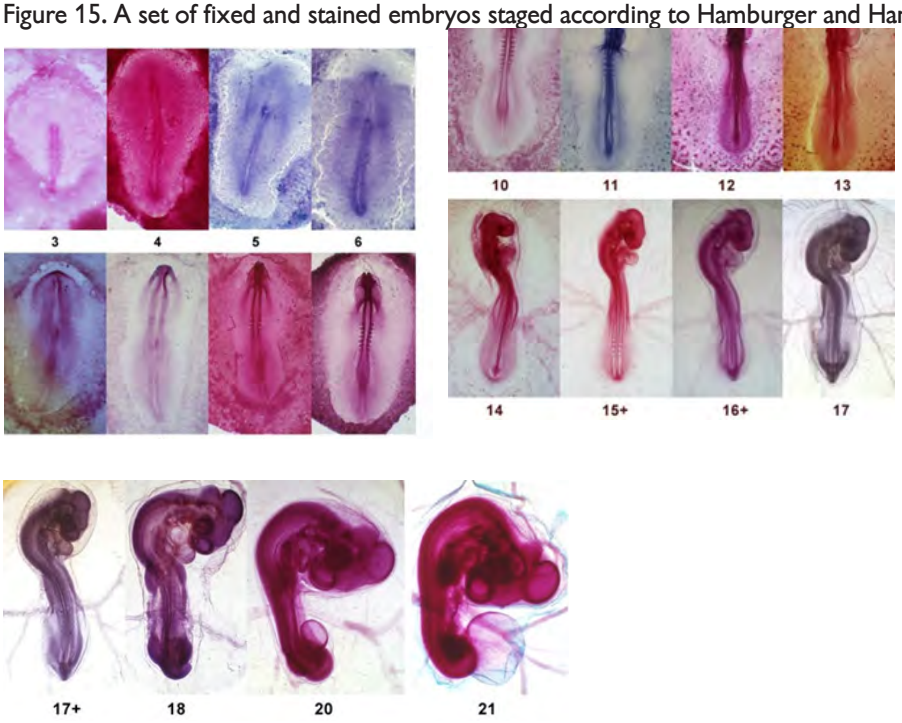
7.1.3.6 Harvest embryo for genomic DNA for sexing. Holding the embryo with tweezers, use a fine pair of scissors to cut the embryo from the vitelline membrane. Place embryo in an Eppendorf tube to be stored at -20°C for sex polymerase chain reaction (PCR). The rest of the embryo and egg can be discarded into the yellow bag. Scissors and tweezers are wiped down in between samples using a blue roll with 70% ethanol.

7.1.3.7 Carefully remove the needle and dispose in the sharps designated bin.

7.1.3.8 Waste and all non-experimental and experimental eggs are double bagged in yellow bags, tagged and placed in the yellow bin in the cold room for incineration or disposed to local sanitary standards..	
7.1.3.9 Aspirating tube is washed in tap water, sprayed with 70% ethanol, allowed to air-dry and then stored in the personal dissection kit.	

A set of fixed and stained embryos staged according to Hamburger and Hamilton (1951) is presented below.

Figure 15. A set of fixed and stained embryos staged according to Hamburger and Hamilton (1951).

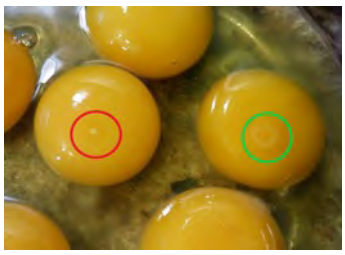


7.2 Isolation of PGCs from chicken blastoderm

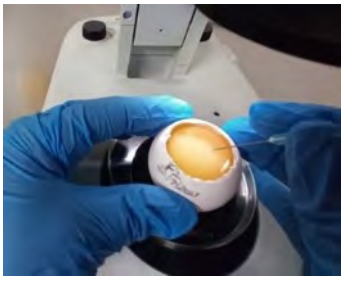
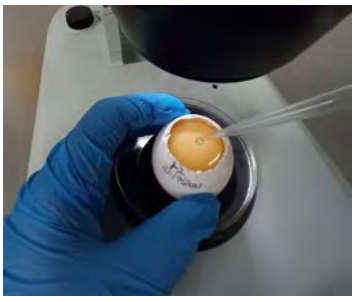
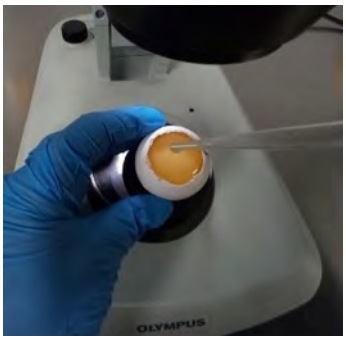
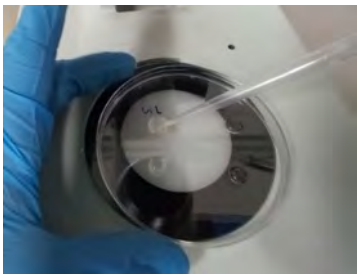
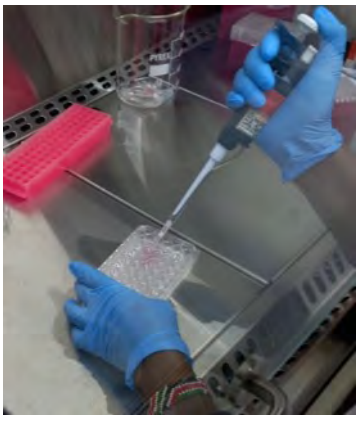
The objective here is to remove the blastodisc from Day 1 embryos following 4–6 hours of incubation for derivation of PGC cultures with or without cryopreservation of tissue. The first part of the process consists of the removal of the blastodisc.

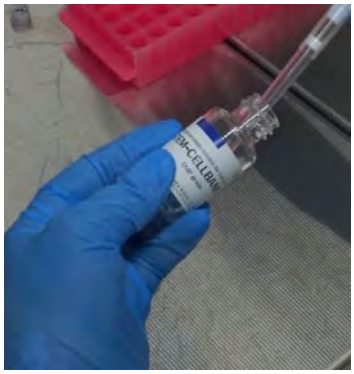
Figure 16. Egg fertility assessment.

Green = fertile, Red = infertile. The little white dot is called the blastodisc. A blastodisc would become a blastoderm if it were fertilized[3] .

7.2.1 Incubate fertile eggs for 4–6 hours at 38°C.I	
7.2.2 Take one egg from the incubator. Lightly spray egg with ethanol and create a window. The blastodisc should be visible on top of the yolk or alternatively break the shell into two halves, pass the egg back and forth to break up the albumen. Drop the albumen in a plastic weighing boat.	
7.2.3 Stroke the surface of the yolk with a spoon or blunt forceps to bring the blastoderm to the top.	

3 https://steemit.com/homesteadersonline/@squishysquid/are-my-eggs-fertile-is-the-rooster-doing-his-job-how-to-tell

7.2.4 Puncture the perimeter of the blastoderm with a sterile needle to make a circle.	
7.2.5 Add drops of Phosphate-buffered saline (PBS) onto the surface to help isolate the blastoderm from the surrounding membranes.	
7.2.6 Carefully suck the blastoderm into the tip of a 1 ml pipette.	
7.2.7 Wash blastoderm briefly in PBS then transfer to 20 µl PBS in a sterile 1.5 ml centrifuge tube.	
7.2.8 Allow the tissue to equilibrate then break up the blastoderm in 300 µl FAOT (FGF2, Activin A, ovotransferrin) and initiate culture in a 48-well plate.	

7.2.9 Alternatively, freeze directly in Stemce llbanker at -80°C and then store in liquid nitrogen (-196 °C).	

7.3 Appropriate timing of PGC collection

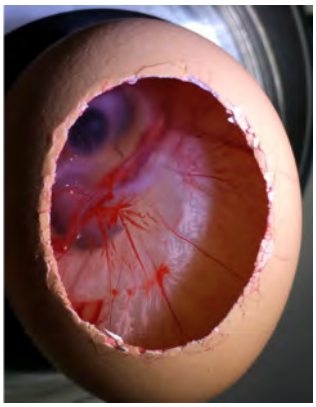
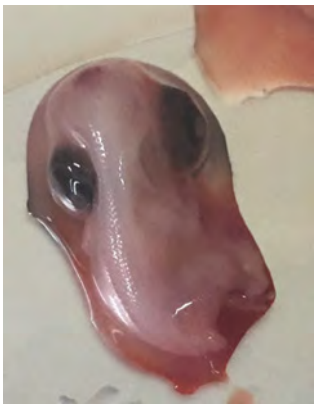
The entrance of PGCs from the anterior part of the extra-embryonic region into the vascular network starts at stage 10 (Hamburger and Hamilton) and is completed in stage 13. Both male and female PGCs collected from 5 to 9-day-old chicken embryos (stages 27–31) can differentiate into functional gametes following transplantation. Male germ cells obtained from adult chicken testes retain germline competency after transplantation to surrogate embryos, but their competency seems lower than that of PGCs. In contrast to males, the germline competency of female germ cells is readily lost from 15.5 days post incubation owing to the start of meiosis. Taking these factors into consideration, the appropriate times for chicken PGC collection and transplantation are stages 13–14 (48-53 hours) as well as 27–31 (5 – 9days) and stages 13–16 (48-56 hours), respectively.

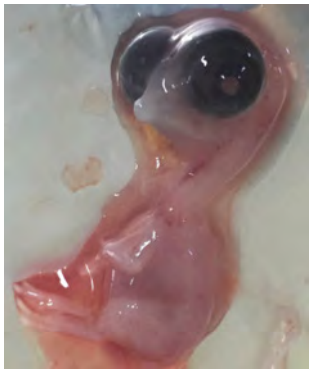
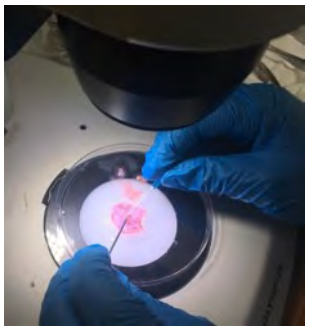
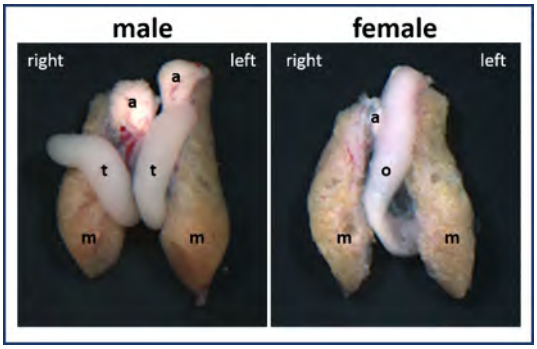
8. Biobanking of chicken embryonic reproductive tissues

8.1 Nine-day incubation of chicken eggs before dissection of chicken gonadal tissues

If dissection of gonadal tissue is scheduled for the morning, the eggs should be set up in the morning on the day of incubation so that they will be at the expected developmental stage.

8.2 Dissection of gonadal tissues

8.2.1 Open egg's shell at the blunt end using forceps and break shell membrane until embryo body is visible. The picture on the right shows the position of the embryo in the egg.	
8..2.2 Pick out the embryo and put in a petri dish. Cull the embryos using the Schedule 1 method by disconnecting the neck using forceps or scissors.	

8.2.3 Under a dissection microscope, position the embryo body so that its stomach is facing upwards. Cut open embryo using scissors to expose the internal organs. Push organs towards cranial direction to expose the gonads and mesonephros.	
8.2.4 Gently dissect both gonads off the mesonephros using 23 G (3.2 cm in length) hypodermal needle. Pick up the gonads using a needle and transfer them to DMEM medium which was pre-dropped at margin area on the same petri dish to wash off extra blood.	
 Light microscopy images of gonads from E19 (14 days) male and female chick embryos. In the male embryo the two testes have a 'tubular' morphology and are of similar size. In the female, the left ovary is a larger structure with a 'ribbon-like' morphology (the right ovary is regressing)[4]. Image caption: [t = testis; o = ovary; m = mesonephros; a = adrenal].	
8.2.5 Transfer the gonadal tissues into a 1.5 ml Eppendorf tube (screw top) containing 500 µl cold DMEM medium and keep on ice until all gonadal pairs are collected.	

8.3 Cryopreservation of whole gonadal tissues

The discovery and elucidation of the function of cryoprotectants, such as glycerol, propylene glycol and methanol, opened the doors for further development of germplasm cryopreservation technology. Generally, the materials being

4 https://www.ed.ac.uk/roslin/chicken-embryology/images

cooled are composed of biological cells, intracellular (within the cell) buffers and cryoprotectants dissolved in extracellular (outside the cell) buffer. Studies of cryopreservation technology seek to quantify the biological, chemical and biophysical changes that occur during the processes of cooling and warming (thawing). Specifically, these studies investigate:

The exchange of water, cryoprotectant and ions across the cell membrane (a semi-permeable membrane that allows only certain substances to pass through while keeping others in or out)

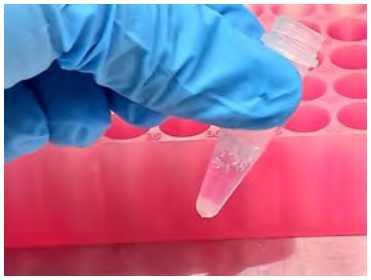
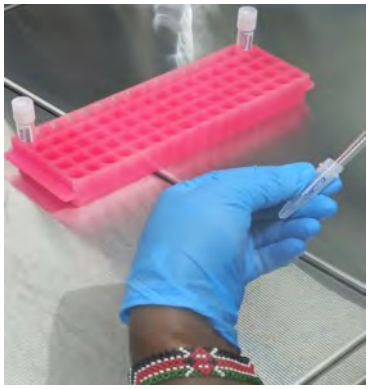
i. Cell size and structure changes

ii. Intercellular or extracellular changes with cryoprotectant addition or removal

iii. Extracellular or intracellular ice formation and heat transfer and

iv. Effects of cooling and warming rates.

Studies of a wide range of single cells, such as sperm and blood cells (including erythrocytes, lymphocytes and hemopoietic cells), uncovered the mechanisms responsible for cell injury during cryopreservation (Pegg 2002). The cryopreservation procedure of embryonic gonadal tissues is described below.

8.3.1 Place the Eppendorf tube in a centrifuge and spin it for four seconds to separate the gonadal tissues from the DMEM medium.	
8.3.2 Gently remove the DMEM medium supernatant without disrupting the gonadal tissues at the bottom. Add 100µl stem cellbanker to the tube for medium exchange and spin the tube as per Section 8.3.1 to collect the tissue at bottom of the tube.	
8.3.3 Gently remove the supernatant and add 200 **µl** of stem cellbanker to the tissue. Transfer all contents into a well labelled cryovial.	

8.3.4 Leave gonadal tissue in stem cellbanker at room temperature for 15 min to equilibrate. Place tubes into a Mr. Frosty™ freezing container pre-equilibrated at -20°C and place in a -80°C freezer overnight.	
8.3.5 The following day, transfer the tubes into -196°C liquid nitrogen and record the storage positions.	

9. Purification, enrichment and long-term culture of PGCs

Recent investigations of several cell surface antigens, such as stage-specific embryonic antigen-1 (SSEA-1) and embryonic mouse antigen-1 (EMA-1) (Karagenç et al. 1996), enabled enrichment of chicken PGCs by magnetic–activated cell sorting (MACS) or florescence-activated cell sorting (FACS) (Mozdziak et al. 2005; Kim et al. 2004). The antigen-antibody reaction-based system allows for isolation of PGCs from both blood and gonads. A unique PGC enrichment method from gonadal tissues was developed in the chicken using the biological property that PGCs are discharged when embryonic gonads are incubated in Dulbecco's phosphate-buffered saline (D-PBS) without Ca2+ and Mg2+ (Nakajima et al. 2001). This is the simplest method currently available to harvest chicken PGCs and is potentially applicable to various avian species. In future, further improvement of the unstable PGC recovery rate is expected.

Chicken PGCs can be propagated in vitro for the long term while maintaining lineage specificity and germline transmission competency (van de Lavoir et al. 2006). Chicken PGCs isolated from embryonic blood can be expanded in a complex medium containing chicken serum, fetal bovine serum (FBS), fibroblast growth factor 2 (FGF2) and buffalo rat liver (BRL) cell-conditioned medium on a feeder of either BRL cells or Sandoz inbred mouse-derived thioguanine-resistant and ouabain-resistant (STO) fibroblasts.

More recently, McGrew and colleagues from the Roslin institute defined serum-free, feeder-free and physio-chemically permissive culture conditions for chicken PGCs, ascertaining that FGF2, insulin and activin are sufficient for the propagation of chicken PGCs (Whyte et al. 2015). Furthermore, a lower osmolality condition (250 mOsm/kg), one of the characteristics of the defined culture system, also enabled efficient derivation and propagation of both male and female chicken PGCs. Establishment of long-term culture systems of chicken PGCs provides the opportunity to significantly amplify donor PGCs before cryopreservation (Nandi et al. 2016) as well as to manipulate the genome with subsequent cloning (Schusser et al. 2013; Park et al. 2014; Oishi et al. 2016) in a manner similar to mouse embryonic stem (ES) cells in the chicken. Further investigations of culture conditions of non-chicken avian PGCs may help in the conservation of chicken genetic resources.

10. Primordial germ cell sample storage and reuse

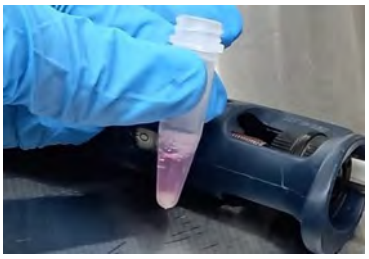
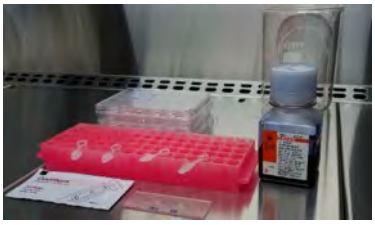
10.1 Ultra-low temperature preservation of PGCs

Ultra-low temperature preservation of chicken PGCs is done using a slow-freezing method. The most widely used freezing protocol is cryopreservation of chicken PGCs in serum containing media supplemented with 10% dimethyl sulfoxide (DMSO) as a cryoprotectant at a cooling rate of -1°C/min until media reaches -80°C. This freezing protocol yields about 50% recovery rate and over 85% viability of post-thawing chicken PGCs. However, using a medium containing more than 10% serum and either 5–10% DMSO or 10% ethylene glycol as cryoprotectants and a cooling rate of -2°C/min, results in higher recovery rates and viability of post-thawed chicken PGCs compared to classic freezing protocols. Commercially available serum and DMSO-based cryomedium also result in higher recovery and viability of chicken PGCs after thawing than the usual freezing protocols. Due to its wider availability and higher performance, the commercial cryomedium CELLBANKER1 (Nippon Zenyaku Kogyo, Koriyama, Japan) has been used to cryopreserve PGCs from chicken.

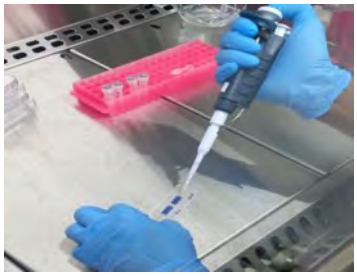
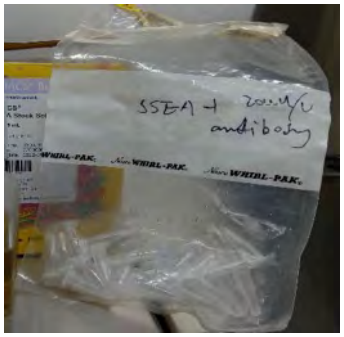
In contrast to the slow-freezing method, a vitrification method can be used to freeze cells to cryogenic temperatures in the absence of ice. Despite a previous study reporting lower recovery and viable rates of vitrified PGCs than in frozen-thawed PGCs, the vitrification method could improve the recovery and viability of chicken PGCs if the protocols are optimized.

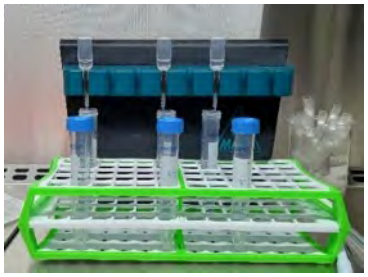
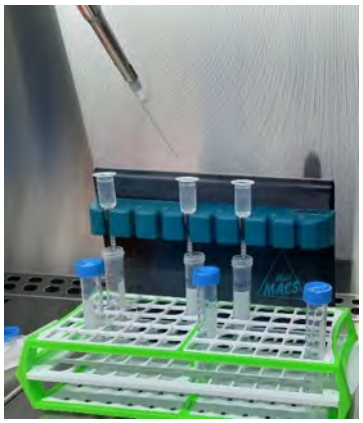
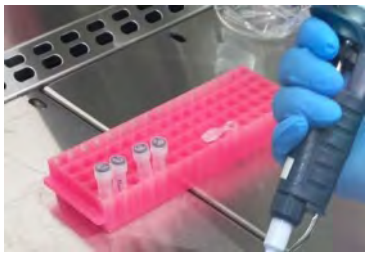
10.2 Preparation of single cell suspension from frozen gonadal tissue

10.2.1 Retrieve the gonadal tissues from liquid nitrogen and place in a warm (37°C) water bath.	

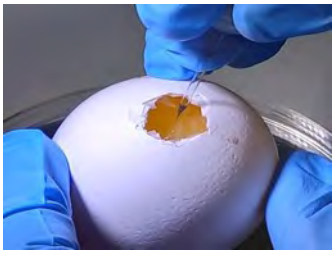
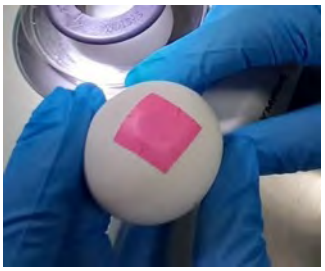
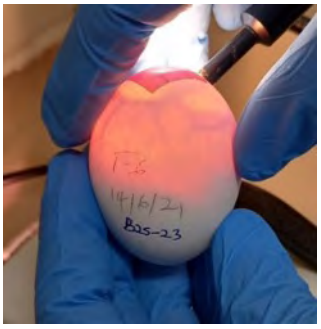
10.2.2 Thaw the tissues at 37°C for 30 seconds.	
10.2.3 In a biosafety hood, remove freezing medium gently, avoiding aspiration of the tissues and slowly add 500 µl DMEM (one drop at a time) to wash and equilibrate the tissues.	
10.2.4 Briefly spin the tubes so that tissues can settle at the bottom. Remove the wash medium and add 200 µl dispase/collagenase solution.	
10.2.5 Incubate the tube at 37°C for 10 min to dissociate the tissues. Flip the tube three times during incubation to suspend the tissues in the dispase/collagenase solution.	
10.2.6 Pipette the tissues and discharge them back into the tube repeatedly to release single cells using the P200 pipette until tissue clumps disappear. Centrifuge at 2,000 rpm for 4 min to pellet the cells.	
10.2.7 Remove supernatant and resuspend all the cells in 500 µl B27 medium. Pellet cells by centrifuging at 2,000 rpm for 4 min.	
10.2.8 Wash the cells twice with 500 µl B27 medium both times and count cells. Resuspend cells in B27 medium. Expected cell density is 10,000 – 15,000 cells/µl.	

10.3 Enrichment of female gonadal PGCs by MACS using SSEA-1 antibody procedure

10.3.1 Resuspend cells obtained in 10.2.8 above in an appropriate volume of MACS buffer to achieve a cell density of 2 x 10^7 cells/ ml.	
10.3.2 Add SSEA1 antibodies to cell suspension at 1 μg/10^7 cells. Incubate at 4°C on roller for 20 min.	
10.3.3 Pellet cells by centrifugation at 2,000 rpm for 4 min. Wash twice with 200 μl MACS buffer.	
10.3.4 Resuspend cells in an appropriate volume of MACS buffer to achieve a cell density of 1 x 10^8 cells/ml.	
10.3.5 Add anti-mouse lgM-conjugated MACS beads using a ratio of 20 μl per 10^7 cells. Incubate at 4°C on roller for 20 min.	
10.3.6 Add up to 1 ml MACS buffer to cells and centrifuge at 2,000 rpm for 4 min to separate cells and microbeads.	

10.3.7 Mount LS column onto magnetic station and set a 15 ml falcon tube to collect flow-through.	
10.3.8 Equilibrate the LS column with 3 ml MACS buffer and load the cell suspension to the column. Put a new 15 ml falcon tube to collect flow-through.	
10.3.9 Wash the column with 3 ml MACS buffer twice. Take the column off the magnetic station and add 3 ml MACS buffer to the column. Insert a plug in and elute the cells into a 15 ml falcon tube.	
10.3.10 Aliquot the elution into 1.5 ml Eppendorf tubes and centrifuge at 2,000 rpm for 4 min to pellet the cells.	
10.3.11 Resuspend and combine all the cells in 200 µl B27 medium, count the cells and pellet the cells by centrifuging at 2,000 rpm for 4 min.	
10.3.12 Wash the cells twice with 200 µl B27 medium per wash, resuspend cells in B27 medium at cell density of 1,000 cells/µl.	

10.4 Injection of cells into host embryos: Production of germline chimeras through PGC transplantation

10.4.1 Host eggs should be set in a rocking incubator (60% humidity), 2.5 days before injection to allow embryos to develop to HH stage 15-16.	
10.4.2 On injection day, add Fast green solution to the cell suspension using a ratio of 1 µl Fast green per 50 µl cell suspension.	
10.4.3 Add 25 mM B/B[5] drug using a ratio of 1 µl drug per 50 µl cell suspension (when iCaspase 9 transgenic hosts are used).	
10.4.4 Aspirate 1 µl of the cell suspension using a mouth pipette and inject into the host through the central aorta.	
10.4.5 Seal the window on the eggshell using paper or silky tapes and return the embryos into an incubator for further development.	
10.4.6 Open the host embryo at 11 days post injection (embryos at 14 days of incubation). Expose the gonads and mesonephros region.	
10.4.7 If fluorecent PGCs are used, examine the GFP[6] and TPZ[7] PGCs in host gonad under the fluorescence microscope to evaluate the re-migration of donor cells.	

5 B/B drug is an homodimerizer, an inducible drug to kill endogenous PGCs. This is a cell-permeable ligand used to dimerize FK506-binding protein (FKBP) fusion proteins and initiate biological signalling cascades and gene expression or disrupt protein-protein interactions

6 Green Fluorescent Protein (GFP) labelled embryos are transgenic embryos with ubiquitous expression of GFP, including PGCs

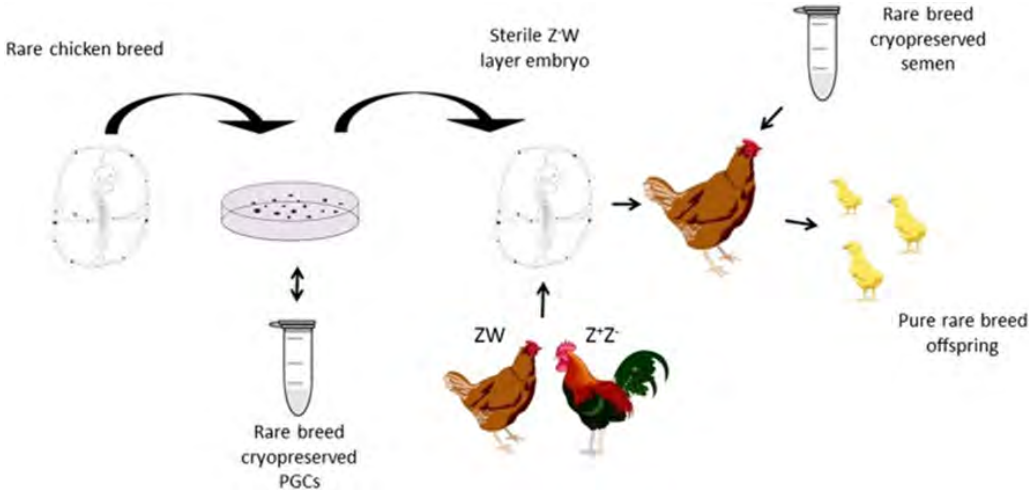
7 TPZ are red fluorescence transgenic embryo, same as GFP birds

11. Primordial germ cell injection into sterilized surrogate embryos

The objective is to microinject avian PGCs into early-stage embryos. Embryos are then called germ line chimeras and can be incubated/hatched to observe germ cell development or bred to generate offspring derived from the injected germ cells.

Woodcock et al. (2019) demonstrated the power of using sterile surrogate hens for reconstituting chicken breeds from frozen material. When cryopreserved PGCs of a rare breed female are introduced into a sterile surrogate hen, which is then inseminated with frozen rare breed semen, 'pure' rare breed offspring are produced as shown in Figure 17.

Figure 17. Diagram showing the reconstitution of a poultry breed using cryopreserved cells.



This approach demonstrates the benefits of using genome editing technology to generate surrogate chickens that could be utilized to preserve the genetic diversity of African chicken breeds. Since cryopreservation of poultry semen has variable success rates across chicken breeds, researchers at the Roslin institute developed sterile male and female (sire and dam) surrogates and simultaneously generated offspring that were homozygous for any genomic variant introduced into the PGCs through genome editing.

12. Cryopreservation of poultry PGCs for restocking programs

Compared with conservation methods that aim to store gametes, cryopreservation of PGCs is advantageous in that each individual cell has the potential, when transplanted into a surrogate, to generate gametes for the lifetime of that individual. The breeding stock of these surrogates can be used to establish breeding programs for rare poultry species and potentially produce enough chicks for restocking programs. In the context of conservation and dissemination of elite or locally adapted breeds, the use of germ cell surrogacy is best suited to producing large numbers of offspring because by selecting highly prolific surrogates, the number of chicks produced can be greatly increased compared with the low reproductive capacities of the local donor species. The application of surrogacy is best suited to support large-scale poultry production in Africa.

13. Conclusions and perspectives on African poultry PGC banks

Both in situ and ex situ conservation strategies can benefit from reproductive technologies, such as artificial insemination, gamete micromanipulation, cell and tissue cryopreservation, in vitro culture and grafting. In addition, these techniques can also be used to obtain data on the reproductive physiology of poultry species. The poultry germplasm banks can be complemented with the preservation of blood and other biological material that can be used for the application of other biotechniques to preserve species.

13.1 Potential benefits of biobanking of poultry genetic resources

A reserve of cryopreserved poultry germplasm will be available for public and private breeders and other researchers to improve management and productivity of African poultry and to ensure sufficient genetic diversity is available to reconstitute lost populations. A biorepository collection will provide a ready source of research material to enhance knowledge on disease resistance, reproductive biology, growth and other traits that can be used to enhance productivity and management tools. Metadata information gathered while collecting germplasm samples will be used to locate breeds and quantify breed resources and productivity within a specific country. This information will be accessible via the internet to guide decisions. Hence, the diverse beneficiaries of CTLGH's biobanking effort include livestock breeders, researchers reconstituting populations and performing various types of molecular studies and all Africans who need a healthy and secure food supply.

13.2 Anticipated outcomes

Anticipated products include:

- A ready supply of preserved germplasm for all major poultry breeds in contributing African countries.
- An information system providing an inventory of preserved germplasm (and other tissues), phenotypic parameters and census data on live populations.
- A national system comprising of agricultural research systems, universities and poultry industry collaborators who prioritize, facilitate and guide collection of information on poultry.
- A more comprehensive understanding of genetic diversity within African chicken breeds.
- Improved cryopreservation protocols that expand CTLGH's capacities to collect and store and restore poultry germplasm.

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Standard operating procedures

Reagents and media preparation

$CaCl_2$ (100 mM)

$CaCl_2.2H_2O$ (Mw = 147.02)	0.147 g
ddH_2O	10 ml
Filter and aliquot 100 µl/tube and store at	-20^{0}C.

Sodium pyruvate (100 mM)

Sodium pyruvate (Mw = 110.04)	0.11 g
ddH_2O	10 ml

Filter and aliquot 500 µl/tube and store at -20^{0}C.

20% Ovalbumin (100×, 5 ml)

Albumin from chicken egg white	1 g
(Ovalbumin, Sigma A5503)	
AKODMEM	5 ml

Put on roller for ≈20 min. If albumin is not completely dissolved, put it back on the roller for an additional 15 min.

Filter and aliquot 550 µl/tube and store at 4^{0}C.

Sodium heparin stock (50 mg/ml, 500×)

Sodium heparin (Sigma H3149)	0.25 g
AKODMEM	5 ml

Filter and aliquot 110 µl/tube and store at 4^{0}C.

ß-mercapotethanol (50 mM, 500×)

ß-mercapotethanol	25 µl
(Sigma M-7522, 14.3M)	
ddH_2O	7.15 ml

Filter and aliquot 110 µl/tube and store at -20^{0}C. Aliquots are thawed and used immediately. Do not reuse.

h-Activin A (25 µg/ml)

20% Ovalbumin solution	50 µl (final concentration 0.1%)
TC grade water	10 ml

Filter through 0.2 µm filter unit.

Spin glass Human Activin-A (from Hi-5 insect cells, Peprotech 120-14 5 µg), disinfect the vial by ethanol wiping.

In the hood, add 200 µl 0.1% ovalbumin to the Activin vial. Allow to sit for several minutes. Mix by pipetting.

Aliquot 25 µl/tube and store at -80°C.

h-FGF2 (25 µg/ml)

Recombinant human FGF	100 µl (25 µg)
(R&D Systems, 234-FSE-025 25 µg)	
TC grade sterile water	900 µl

Do not filter. Aliquot 20 µl/tube. Store at -80^{0}C.

Note: The product is shipped on dry ice as a 100 µl solution containing Tris-NaCl and BSA as a carrier. It should be diluted and frozen directly. The final concentration of BSA in the diluted frozen aliquots will be 0.25%, with 100 mM NaCl. This should be sufficient carrier protein for the stable long-term storage of these aliquots.

Ovotransferin (10 mg/ml)

Conalbumin from chicken egg white	0.05 g
(Ovotransferin, Sigma C7786)	
AKODMEM	5 ml

Filter and aliquot 25 µl/tube. Store at 4^{0}C.

hBMP4 (25 µg/ml)

hBMP4 (Peprotech 120-05)	25 µg
Citric acid (10 mM, sterile)	1 ml

Do not filter, aliquot and store at -80°C.

Modified DMEM (50 ml)

DMEM (Gibco, Cat No. 21068-028)	50 ml
Sodium pyruvate (100×, 100mM)	700 µl (final concentration 1.4×, after dilution in AKODMEM, it is 1×)
(or Gibco 11360-039)	

AKODMEM (Avian KO DMEM, to replace the custom ordered DMEM 50 ml)

Modified DMEM (from above)	38 ml
Tissue culture grade water	11.6 ml
MEM vitamin solution	500 µl
(100×, Gibco 11120052)	

The osmolarity should be 255 mOsm/kg. Store at 4°C.

Option 1 (needs to be tested):

DMEM (Gibco, Cat No. 21068-028)	38 ml
Tissue culture grade water	11.6 ml
Sodium pyruvate (100×, 100 mM)	500 µl (532 µl)
(or Gibco 11360-039)	
MEM vitamin solution	500 µl
(100×, Gibco 11120052)	

The MEM vitamin solution should be aliquoted and stored at -80°C.

AKODMEM-B27 Stock (50 ml)

AKODMEM	47 ml
B27 supplement	1 ml
(50×, Gibco 17504-044)	
GlutaMAX-I (100×, 200 mM)	0.5 ml
MEM NEAA (100×)	0.5 ml
EmbryoMax® ES Cell	0.5 ml
Qualified nucleosides (100X, ES-008-D)	
Sodium pyruvate (100 mM)	0.2 ml
ß-mercapotethanol (50 mM, 500×)	0.1 ml
CaCl2 (100 mM)	75 µl
20% Ovalbumin (100×)	0.5 ml
Sodium heparin (50 mg/ml, 500×)	0.1 ml
Pen/Strep (100×) (optional)	0.1 ml

Filter to sterilize and store at 4°C for **<5 weeks, protect against the light.**

Note:

1. Aliquot nucleosides 510 µl/tube and store at -80C. Prior to use, ensure it is mixed well and discard whatever is left in the tube to avoid freeze-thawing.
2. No antibiotic use is preferred.
3. B27 is sensitive to light.
4. There is no need to filter.

FAOT (for all chicken PGC cultures, serum free)

AKODMEM-B27 stock	5 ml
h-Activin A (25 µg/ml)	5 µl
h-FGF2 (25 µg/ml)	0.8 µl
Ovotransferin (10 mg/ml)	5 µl

Store at 4°C for **<1 week** and protect from light. Do not filter otherwise the growth factors in the medium will be compromised.

Freezing Solution (2×)

AKODMEM	4.1 ml
DMSO	400 µl (final concentration 8%)
Chicken serum (γ-irradiated)	500 µl (final concentration 10%)
CaCl2 (100 mM)	7.5 µl

Freshly prepare the freezing solution and filter to sterilize.

Note for medium preparation:

1. All stocks should be used within 6 months.
2. All frozen stocks (except B-27) should be stored at 4°C after first use and must be used within 30 days.

Derivation of PGCs from blood

Egg preparation

Note:

- After laying, the eggs should be stored at 15°C for a maximum of ten days or at room temperature for one week. The eggs will not develop properly if they are left in storage for too long.
- For PGC derivation, the eggs are supposed to be at stage 16–17 (56 hours in incubation). Earlier stage eggs can also be used if the heart is beating. However, older stages (more than 17) are not recommended because in practice old embryos have a lower success rate.
- The blood can be sucked from leaked blood or peripheral blood vessels.
- The needle can be used for several eggs receiving the same cells, but should be changed when moving to different cells to avoid contamination.
- The PGC remains viable at room temperature and high pH (around 8). Therefore, during derivation PGCs can be left at room temperature.

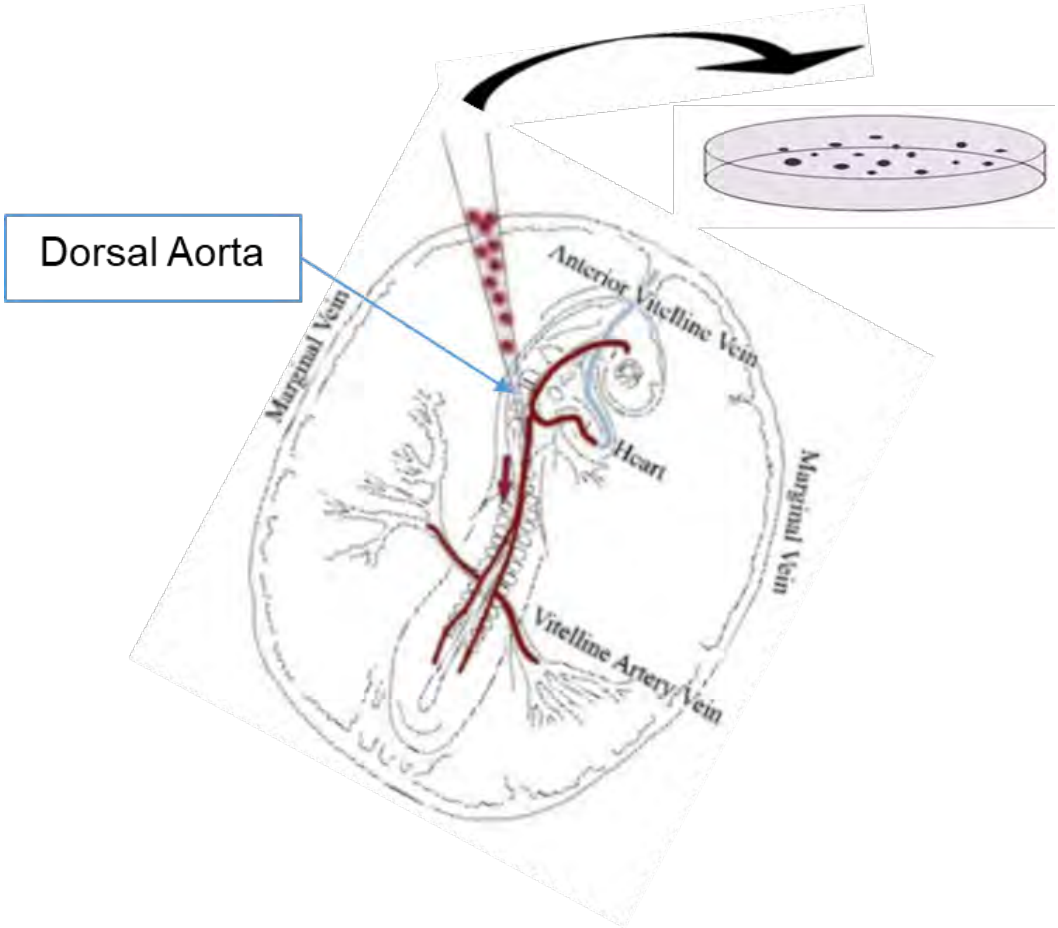
Procedure

1. On Monday morning at 900 hours, put the eggs in the incubator without rocking.
2. On Wednesday afternoon at 1700 hours (56 h), remove the eggs from the incubator and leave them overnight at room temperature.

3. On Thursday morning, put the eggs into the incubator for 2 hours to warm up before PGC derivation. Embryos should be around stage 16 but not older than stage 17.
4. Prepare a 48 well plate by **adding sterile dH_2O into the wells of the surrounding rows and columns to maintain the humidity in the plate during culture.** If this is not done, the PGC will not grow well.
5. Add 300 µl of FAOT into each well and leave the plate in the hood.
6. Under the microscope, break the capillary end using ethanol sterilized forceps and insert capillary end into the aspiration tube. Drape the aspirator apparatus over the microscope so that both ends do not touch laboratory surfaces. Maintain this positioning throughout the whole procedure.
7. Take one egg from the incubator. Lightly spray the egg with ethanol and make a small opening or window. The heart of the embryo should be visibly beating.
8. Blow bubbles onto the egg surface using a capillary to check that the needle is open. Insert the capillary into the dorsal aorta at a shallow angle. Aspirate 1–2 µl of embryonic blood into the capillary.
9. In the hood, blow the blood sample into one or two wells (blow the blood in 20 µl AKODMEM-B27 stock in an EP tube and split into 2 wells) of a 48-well tissue culture plate. Repeat for up to 20 embryos.
10. Harvesting embryo for genomic DNA for sexing: Holding the embryo with tweezers, use a fine set of scissors and separate the embryo from the vitelline membrane. Place embryo into an Eppendorf tube to be stored at -20°C for sex PCR. The rest of the embryo and egg can be discarded into a biohazard bag. Scissors and tweezers should be wiped down using blue roll with 70% ethanol between samples.
11. Carefully remove the needle and dispose in sharps' bin.
12. Wash the aspirating tube in tap water, spray with 70% ethanol, allow to air dry, then store in the personal dissection kit.
13. Feed the cells every two days by removing 90 µl of medium from the side of the well and add 100 µl of the new medium to each well.
14. When the well is confluent, transfer the cells into a 24-well plate (500 µl) by centrifuge.
15. Feed the cells every two days by removing 300 µl medium from the side of well and adding 350 µl of the new medium.
16. When the cells are confluent, split them into two wells (1:2) then feed as standard.
17. When the cells are confluent, freeze them into four tubes. Normally, there are about 400,000 cells when a 24 well plate is confluent. The cells could be frozen with as little as 50,000 cells in each tube.

	Monday	Wednesday	Friday
48-well plate	Remove 90 µl and add 100 µl of new medium		
24-well plate	Remove 300 µl and add 350 µl of new medium		

Figure 18. Dorsal aorta dissection.



Derivation of PGCs from embryo blastoderm

Note: Sometimes, the eggs are not of good quality and cannot develop to Stage 16–17. To avoid loss of eggs, the PGC could be derived from the embryo disc at a very early stage (1–3).

Egg preparation

- Incubate the eggs as routine for 4–6 hours.

Procedure

Note: This is better done in a hood.

1. Prepare sterile PBS.
2. Prepare a 96-well plate with 100 µl of FAOT per well depending on the number of eggs.
3. Prepare a 6 well plate with 3 ml PBS in each well.
4. Disinfect the egg with 70% ethanol
5. Break the egg to expose the embryo
6. Under the microscope, use a 15-gauge needle (Sigma HSWNH15112-100EA) to cut the embryo disc by penetrating the membrane around the embryo disc.
7. Use a plastic Pasteur pipette to add a few drops of sterile PBS on top of the embryo disc. Leave it for 1 min. The embryo disc will float to the top above the yolk.
8. Use a cut plastic Pasteur pipette to suck the embryo disc and transfer to the 6-well plate (each well can take 3–4 embryo discs).

9. Repeat the same until 3–4 embryo discs are collected.
10. Under the microscope, use fine forceps to remove the embryo discs from the yolk carefully.
11. Transfer the embryo disc to a prepared 96 well plate.
12. After all the embryo discs are transferred to a 96 well plate, use P100 or P200 pipettes to dissociate the embryo disc.
13. Culture the cells at 37°C and 5% CO2.
14. Please note that there are many other cell types in the embryo disc and many cells will attach to the bottom. After some time, transfer the cells in suspension to a new well several times (until pure cell lines are established) to eliminate other cell types.
15. Since PGCs settle at the bottom, wash the bottom gently and transfer the supernatant to an EP tube, centrifuge resuspend in the new medium and plate in a new well.
16. The other steps are the same as per the derivation from blood procedure described above.

Freezing PGCs

Note: DMSO is very toxic to PGCs, therefore, quick execution is recommended. Add DMSO solutions slowly, otherwise PGCs will burst due to osmotic shock. Normally, one well can be frozen in four tubes. Each tube should contain ≥50,000 cells that can be recovered in a 24-well plate.

Procedure

1. Prepare freezing medium.
2. Pre-label four Nunc 1.8 ml cryovials and place them in the hood.
3. Place freezing box in 4°C fridge for 30 min.
4. Both PGC media and PGC media containing DMSO must be kept at room temperature.
5. For freezing of one well of a 24-well plate, PGCs confluent (≈200,000 cells).
6. Remove all cells from well.
7. Spin at 1,600 rpm for 4 min.
8. Resuspend cells in 500 µl of PGC AKODMEM-B27 stock (or AKODMEM).
9. Add an equal volume of freezing solution (500 µl) to Eppendorf tubes containing cells.
10. Note: Drops of the freezing solution should be added slowly and mixed by flicking the tube.
11. Mix and aliquot 250 µl of PGC mixture to the empty labelled cryovials.
12. Place cryovials in pre-cooled freezing box (4°C) and store in a -80°C freezer.
13. Transfer the freezing box to a -150°C freezer after 24 hours.

Recovering frozen PGCs

Procedure

1. Prepare a 24-well plate by **adding sterile dH_2O into the wells of the surrounding rows and columns to maintain the humidity in the plate during culture**. If this is not done the PGCs will not grow well.
2. Remove PGC vial from freezer.
3. Either thaw in the palm of the hand or in a beaker filled with water at room temperature. **Do not use a water bath as cells should not be heated to 37°C in the DMSO solution.**
4. Add AKODMEM-B27 stock (or AKODMEM) to the cryovial (if volume in cryovial is 250 µl add 0.5 or 1.0 ml) one drop at a time. The medium can be added to the vials to facilitate complete thawing of cells.
5. Transfer the diluted cell suspension to a 1.5 ml Eppendorf tube.

6. Spin cell suspension in a centrifuge at 2,000–2,500 rpm for 10 min. (**Note that the centrifuge force is higher and the time required is longer than when working with non-frozen cells as the frozen cells are more difficult to pellet. Normal centrifuge for fresh PGCs is 1,600–2,000 rpm for 4 min**).
7. Carefully remove the supernatant by pipetting from the inside wall of the Eppendorf tube. Pellet, if visible, will be extremely small. Discard supernatant.
8. Carefully resuspend cells in PGC medium (FAOT) containing growth factor, washing inner surfaces of Eppendorf tube and transfer to a well (500 µl medium for a 24-well plate and 300 µl for a 48-well plate).
9. Allow the plate to sit in the hood at room temperature for 5 to 10 min to equilibrate cells.
10. Place the plate in the incubator.
11. Optional: Check the cells the next day and replace the old medium with new medium with or without the aid of centrifugation.
12. Feed the cells every two days until confluent and then split them at a ratio of 1:2.

Notes for PGC culture

1. It is very important to add water into the surrounding wells of the plate to ensure humidity is maintained on the plate otherwise the PGCs will not grow well.
2. Derive the PGCs from 1 µl of blood in a 48-well plate (300 µl medium). It takes about three weeks for the blood cells to die off and for the PGCs to confluence. Blood cells seem to inhibit PGC growth. Then transfer the cells to a 24-well plate. Feed every two days until confluent, then split the cells at a ratio of 1:2 again into two wells of a 24-well plate. The cells can then be frozen (1:2) when confluent. There will be four tubes of PGCs to freeze.
3. The PGCs remain viable at room temperature and at high pH (≈ 8) thus can be left at room temperature during derivation.
4. Fresh and frozen cells require different centrifuge forces to pellet. Some cells with attached fibroblasts tend to be difficult to pellet.
5. Feed the cells by replacing some of the old medium with new medium. For PGCs that will be injected for germline transmission, replace the medium after centrifugation.

Biobanking of chicken embryonic gonadal tissues

Materials

Fast green solution: 5 mg/ml or 0.5% w/v, 1 in 50 dilution for working concentration

B27 medium

MACS buffer: 0.5% BSA, 2 mM EDTA in PBS

Anti-mouse IgM-conjugated MACS beads

SSEA1 antibody

R&D system, Cat No. MAB2155, mouse anti-Human/Mouse SSEA-1 Antibody, IgM

dispase/collagenase

Stem-cell banker medium

23G (3.2 cm in length) hypodermal needle

Mr. Frosty™ freezing container

Forceps or scissors

Mouth pipette

Glass needle

Procedure

1. **Incubation of chicken eggs**: Nine days before dissection of chicken gonadal tissues.

Note: If dissection of gonadal tissue is planned for the morning, the eggs should be prepared in the morning of the incubation day so that they will be at the expected developmental stage.

2. **Dissection of gonadal tissues procedure:**

2.1 Open the eggshell from the blunt end using forceps and break the shell membrane until the embryo is visible.

2.2 Pick out the embryo and place it in a petri dish. Cull the embryos using the Schedule 1 method and disconnect the neck using forceps or scissors.

2.3 Under a dissection microscope, position the embryo body so that the stomach faces upwards. Cut open the embryo using scissors to expose the internal organs. Displace organs towards the cranial direction to expose the gonads and mesonephros.

2.4 Gently dissect both gonads off the mesonephros using a 23G (3.2 cm in length) hypodermal needle. Using the needle, pick and transfer gonads to the DMEM medium which was pre-dropped at margin area on the same petri dish to wash off extra blood.

2.5 Transfer the gonadal tissues to a 1.5 ml Eppendorf tube (screw top) with 500 µl cold DMEM medium and place on ice until all gonadal pairs are collected.

3. Cryopreservation of whole gonadal tissues procedure

3.1 Place the Eppendorf tube in the centrifuge and spin for 4 seconds. The gonadal tissue will sink to the bottom of the tube.

3.2 Gently remove the DMEM medium supernatant without disrupting the gonadal tissues. Add 100 µl Stem-cell banker medium to the tube for medium exchange and quick spin the tube as in section 8.3.1 to allow gonadal tissue to collect at the bottom of the tube.

3.3 Gently remove supernatant and add 200 µl Stem-cell banker medium to the tissue.

3.4 Leave gonadal tissue in Stem-cell banker medium on ice for 15min to equilibrate the tissue. Place the tubes into a Mr. Frosty™ freezing container and place in a -80°C freezer. (Vitrification of tissues by dropping tube in liquid nitrogen could be tested as well)

3.5 Transfer the tubes into a -150°C freezer the following day.

4. Preparation of single cell suspension from frozen gonadal tissue procedure

4.1 Retrieve the gonadal tissues from the -150°C freezer and place on dry ice.

4.2 Thaw the tissues at 37°C for 30 s until fully thawed.

4.3 In a biosafety hood, remove the freezing medium gently, avoiding aspiration of the tissues. Add 500 µl DMEM slowly (dropwise) to wash and equilibrate the tissues.

4.4 Briefly spin the tubes if the tissues fail to descend to the bottom. Remove the wash medium and add 200 µl dispase/ collagenase solution.

4.5 Leave the tube at 37°C for 10 min to dissociate the tissues, flipping the tube to suspend the tissues three times during incubation.

4.6 Pipette the tissues and discharge them back into the tube repeatedly to release single cells using a P200 pipette until tissue clumps disappear. Centrifuge at 2,000 rpm for 4 min to pellet the cells.

4.7 Remove the supernatant and resuspend all the cells in 500 µl B27 medium. To pellet cells, centrifuge at 2,000 rpm for 4 min.

4.8 Wash the cells twice with 500 µl B27 medium and count cell numbers. Resuspend the cells in B27 medium. The expected cell density is 10,000–15,000 cells/µl.

5. Enrichment of female gonadal PGCs by MACS using the SSEA-1 antibody procedure

5.1 Resuspend cells in appropriate volume of MACS buffer to reach cell density of 2 x 107 cells/ml.

5.2 Add SSEA1 antibodies to the cell suspension at the rate of 1 µg/107 cells. Incubate at 4°C on roller for 20 min.

5.3 Pellet cells by centrifugation at 2,000 rpm for 4 min. Wash twice with 200 µl MACS buffer.

5.4 Resuspend cells in appropriate volume of MACS buffer to reach cell density of 1 x 108 cells/ml.

5.5 Add anti-mouse lgM-conjugated MACS beads at a ratio of 20 μl per 107 cells. Incubate at 4°C on roller for 20 min.

5.6 Add up to 1 ml of MACS buffer to cells. Centrifuge at 2,000 rpm for 4 min to separate cells and microbeads.

5.7 Mount the LS column onto the magnetic station and set a 15 ml falcon tube to collect flow-through.

5.8 Equilibrate the LS column with 3 ml MACS buffer and then load the cell suspension to the column. Put a new 15 ml falcon tube to collect flow-through for safe keeping.

5.9 Wash the column with 3 ml MACS buffer twice. Take the column off the magnetic station and add 3 ml MACS buffer to the column. Insert a plug in and elute the cells into a 15 ml falcon tube.

5.10 Aliquot the elution into 1.5 ml Eppendorf tubes and centrifuge at 2,000 rpm for 4 min to pellet the cells.

5.11 Resuspend and combine all the cells in 200 μl B27 medium. Count the cells and pellet them by centrifugation at 2,000 rpm for 4 min.

5.12 Wash the cells twice with 200 μl B27 medium. Resuspend cells in B27 medium to achieve a cell density of 1,000 cells/μl.

6. Injection of cells into host embryos

6.1 Place host eggs in a rocking incubator (60% humidity), 2.5 days before injection to allow embryos to develop to HH stage 15–16.

6.2 On injection day, add Fast green solution to the cell suspension at a ratio of 1 μl Fast green per 50 μl cell suspension.

6.3 Add 25 mM B/B drug at a ratio of 1 μl drug per 50 μl cell suspension.

6.4 Aspirate 1 μl supernatant using a mouth pipette and inject into the surrogate host through the central aorta.

6.5 Seal the window on the eggshell using paper or silky tapes and return the embryos into the incubator for further development.

6.6 Open the host embryo at 11 days post injection (embryos at 14 days of incubation) and expose the gonads and mesonephros region.

6.7 Examine the GFP and TPZ PGCs in the host gonad under a fluorescent microscope to evaluate the re-migration of donor cells.

Embryo injection and hatching protocol

Embryo preparation procedure

1. Place eggs in the incubator on Friday at 1900 hours with pointed end up.
2. Incubate until Monday 0800 hours.
3. Remove each egg for injection.

Protocol for windowing and sealing manipulated egg

1. Weigh the egg.
2. Wash egg and spray with 70% ethanol.
3. Make a tiny hole on the blunt end of the egg with sharp forceps. Be sure not to penetrate the shell membrane. This will reduce the pressure of the air pocket so that the egg could collapse.
4. Using forceps, make a small window (5 mm – 1 cm diameter) on the pointed end of the egg.
5. Remove 3 ml albumin from the egg using a 1ml syringe. Please note that Tagami et al. (2007) specifies 5 ml albumin but it is recommended that 3 ml is used in these experiments. Monitor humidity and weight loss.
6. Remove 1–2 μl of blood to create sufficient room for injected cells.
7. Optional: Place this blood in the tube for genotyping of the host egg.
8. Inject 1–2 μl PGC solution.
9. Add 100 μl Penicillin-Streptomycin (pen/strep) antibiotic solution (100□).

10. After injection, place a small piece of cling film (double layer) on the egg.
11. Use thin albumin from newly laid egg as glue and use a cotton bud to seal the cling film around the egg.

Incubation of eggs

1. Weigh the eggs again and place into a Brinsea incubator.
2. Incubate eggs with pointed end up.
3. Turn eggs by 45° in the Brinsea incubators every 30 min and maintain the humidity at 60% and temperature at 38.4°C until Day 18.
4. Weigh eggs every two days to monitor water loss (Monday, Wednesday, Friday).
5. Record dead embryos.
6. Candle at Days 10 and 14 and store eggs with dead embryos at 4°C.
7. Move to hatcher at Day 18 maintaining temperature at 37/37.5°C for hatching. According to Tagami et al. (2007), 90% of chicks can break out of the egg whilst the remaining 10% of chicks cannot and require assistance.

Re-injection of PGC into chicken embryos

Embryo preparation

1. Place eggs in incubator on Friday at 1900 hours with pointed end up.
2. Incubate until Monday 0800 hours.
3. Remove each egg for injection (Stage 16–17).

Primordial germ cell migration tests

For the study of PGC migration, 500–1,000 PGCs in 1 µl are injected into each embryo.

Cell preparation

- Pipette all the cells to a centrifuge tube
- Centrifuge at 1,600 rpm for 4 min.
- Remove the supernatant.
- Allow 1 min for the remaining medium to descend completely.
- Measure the volume and top up with ≈ 10 µl of diluted DMEM. Please note that normally 500 – 1,000cells in 1µl are injected into each embryo and 5,000 cells/µl could be injected for transgenic work to compete with host PGCs.
- Add 0.2 µl of food dye (1% Fast green FCF (Sigma F7258-25G) in PBS, filtered to sterilize.

Injection

Injection is ideally done in a hood for sterility as well as to achieve a higher survival rate. Normally, many embryos die after injection.

Phosphate-buffered saline (PBS) with pen/strep should be prepared in advance. Put a few drops using a plastic Pasteur pipette onto the embryo after injection.

- Break the needle under the microscope.
- Strike the cells and suck 1–2 µl into the needle.
- Drape the aspirator on the microscope.
- Spray the egg with 70% ethanol.

- Open the egg and remove part of the shell membrane to expose the embryo. It is better to remove as little membrane as possible to avoid evaporation as the egg needs to continue to develop in the incubator.
- Insert the needle into the dorsal aorta and blow the cells. The blue colour is immediately visible within the blood vessel network. Blow the cells out slowly to avoid too much pressure.
- Put a few drops of PBS (pen/strep) on the embryo.
- Seal the egg with cellulose tape. Ensure that it is properly sealed otherwise the embryo will dry out during incubation.
- Place the egg back into the incubator immediately and keep overnight. The incubator should be humidified with water.
- Next morning, check the surviving embryos and remove the dead ones.
- After 4 days, around Day 6.5 (e.g. Friday - Tuesday), the gonads can be dissected out and checked for PGC migration. Alternatively, wait until Day 9.5 to check the migration.

Germline transmission (transgenic or regeneration of chicken line from frozen PGCs)

Background

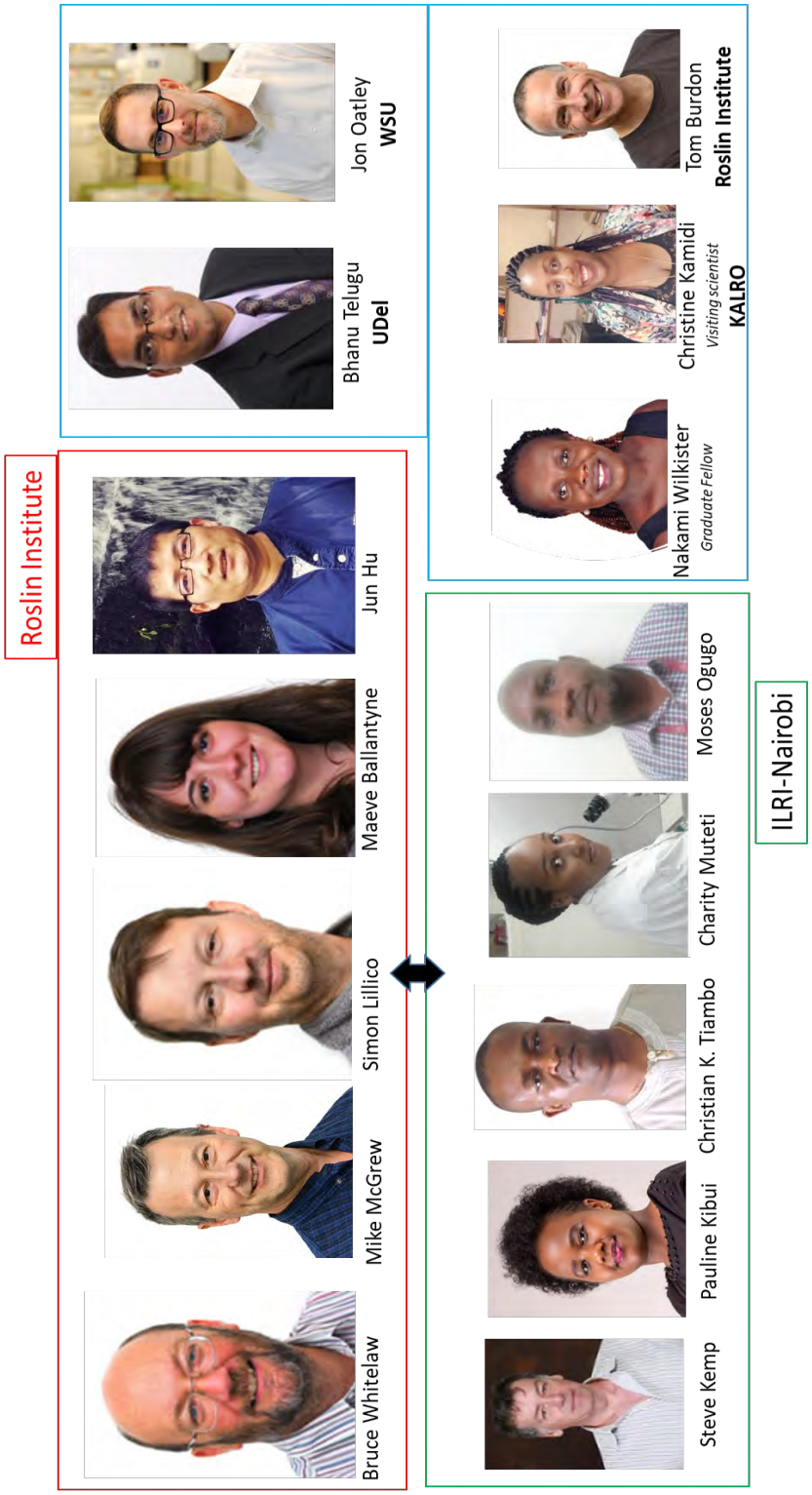
- For optimum yield, it is recommended that many cells (4,000–5,000 PGCs in 1 μl) are injected into each embryo to compete with the host embryo's PGCs.
- For better hatching rates, the egg windows should be small.
- The embryo stage should not exceed Stage 17. If the embryo is too old, its own PGCs will have already migrated and the injected PGCs will not migrate and compete with the host cells as desired.

Cell preparation is the same as above.

Protocol for windowing and sealing manipulated eggs

1. Weigh the eggs.
2. Wash eggs and spray with 70% ethanol.
3. Make a tiny hole on the blunt end of the eggs with sharp forceps exercising caution not to penetrate the shell membrane. This will reduce the pressure of the air pocket so that the eggs can colapse.
4. Using forceps, make a small window (5 mm – 1 cm in diameter) in the eggs at the pointed end.
5. Remove 3 ml albumin from the egg using a 1 ml syringe. Please note that Tagami et al (2007) specifies 5 ml albumin but it is recommended that 3 ml is used in these experiments. Monitor humidity and weight loss.
6. Remove 1–2 μl of blood to create sufficient room for injected cells.
7. Optional: Place this blood in a tube for genotyping of the host egg.
8. Inject 1–2 μl PGC solution.
9. Add 100 μl Pen/Strep solution (100□).
10. After injection, place small piece of cling film (double layer) on the egg.
11. Use thin albumin from newly laid eggs as glue and use a cotton bud to seal the cling film around the egg.

The reproductive technology and precision breeding team



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