Chapter 16

Cryopreservation of Avian Semen

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Abstract

Cryopreservation protocols for semen exist for bird species used in animal production, fancy and hobby species, and wild bird species. Freezing of bird oocytes or embryos is not possible. Cryopreservation of avian semen is used for preserving (genetic diversity of) endangered species or breeds. Freezing semen can also be used in the breeding industry for maintaining breeding lines, as a cost-effective alternative to holding live birds. Success and efficiency of cryopreservation of bird semen differs among species and breeds or selection lines. This chapter describes important variables of methods for collecting, diluting, cold storage, and freezing and thawing of bird semen, notably the medium composition, cryoprotectant used and its concentration, cooling rate, freezing method, and warming method. Media and methods are described for freezing semen using either glycerol or DMA as cryoprotectant, which both are known in chicken and a number of other bird species to render adequate post-thaw fertility rates.

Key words Avian, Poultry, Semen, Cryopreservation, Extender, Insemination

1 Introduction

1.1 Historical Perspective

Poultry spermatozoa were, in fact, the first spermatozoa and arguably the first vertebrate cells to be frozen and thawed successfully with significant post-thaw viability, which was made possible by the serendipitous discovery of the cryoprotective property of glycerol [1]. The fertilizing ability of the frozen fowl semen was not immediately evident due to the contraceptive effect of glycerol. But after introducing the removal of the glycerol by dialysis, the chicks that were then obtained in 1951 were the first vertebrates in the world to be produced from eggs fertilized with sperm preserved by freezing [2, 3]. The cryoprotective action of glycerol and other neutral solutes was explained by Lovelock [4] as being due to the ability of these compounds to permeate through the cell membrane and to reduce the rise of intra- and extracellular electrolyte concentration during freezing. In fact, a great number of similar membrane-permeating compounds can be effectively used as CPA for freezing live cells.

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1.2 Background

Cryopreservation protocols for semen are described in the literature for a number of bird species used in animal production, as well as for fancy and other hobby species and wild bird species.

In birds and other macrolecithal taxa, cryopreservation of the ova or embryos is not possible. If only male gametes (i.e., semen) can be used, it would take at least six generations of backcrossing with frozen-thawed semen to recover a lost endangered breed or a selection line. This makes it laborious and costly, and it is therefore less practical. Collection, cryopreservation, and use (grafting) of avian ovaries [5, 6] or primordial germ cells [7, 8] are potential alternative strategies that are currently being developed. This chapter will only discuss methods for cryopreserving avian semen.

Most studies on freezing of avian semen have been done with domestic bird species. Semen cryopreservation is described for other domestic bird species, including turkey, duck species, goose, and guinea fowl [9]. More recent studies or reviews have been published, e.g., for quail [10], emu [11], guinea fowl [12, 13], and goose [14]. Cryopreservation of nondomestic bird species was reviewed in [15, 16]. More recent studies are, for example, for crane species (e.g., [17]) and pigeon (e.g., [18]).

Success and efficiency of cryopreservation of semen from birds or mammals differs among species and breeds or selection lines. These differences in part relate to intrinsic "freezability" of the semen but also result from practical issues, such as the size of the ejaculate relative to the insemination sperm dosage, and also the way the breeding and animal production industry is organized. In cattle, mostly all breeding relies on artificial insemination with frozen/thawed semen. In bird species such as chicken or Turkey, however, artificial insemination is largely restricted to "fresh" semen.

The fact that frozen/thawed semen is little used in commercial fowl production has also limited the amount of research on cryopreservation methods. One reason for limited use is that the number of breeding units that can be produced per ejaculate is quite small, certainly in comparison with, for example, bovine semen. Wishart [19] showed that approximately 300 million sperm cells per hen must be inseminated to achieve plateau level (>90%) fertile eggs, in contrast to only approximately six million fresh semen. That means that with frozen semen, only 7 hens can be inseminated from a single ejaculate of 2 billion spermatozoa, as opposed to 300 hens when using fresh semen. Another reason lies in the organization of the poultry industry. In cattle, selective breeding is on the level of individual males, whereas it is being executed on breeding line level in chicken. This makes it possible to keep males available on site. In fact, natural mating in group housing is used in grandparent and parent stock for the production of hatching eggs. And if artificial insemination is used, in most cases, this is with semen of males held on site, which can be used fresh/extended.

In the top of the selective breeding pyramid, frozen/thawed semen can be useful. However, although various authors have reported fertility levels of ±90% with frozen/thawed poultry semen [19–22], the possibility of using frozen semen seems underestimated in the poultry industry. Currently, cryopreservation of avian semen does play a role in ex situ gene banking to preserve endangered wild species or for preserving genetic diversity in commercial species (Reviewed in [9]). Main repositories are those of the North American USDA National Animal Germplasm Program [23], the French National Cryobank of Domestic Animals [24], and the Dutch Centre for Genetic Resources, the Netherlands (CGN) [21]. Additionally, many other countries have germplasm repositories of domestic bird species in private companies or public research institutes. In addition to (longer-term) gene banking of endangered breeds, frozen semen can also be important for maintaining breeding lines in research institutes and poultry breeding organizations, as a cost-effective strategy compared with holding and propagating live birds.

1.3 Extenders and Freezing Media

Both for freezing semen and for the fresh use of semen, the diluent medium used is very important. The dilution of semen provides the sperm cells space and access to oxygen and substrates for metabolism. Access to oxygen or to a glycolyzable substrate (depending on the species) is important during semen storage [25–27]. Also a stronger dilution makes that the milieu for the sperm cells does not rapidly deteriorate as a result of products of cell metabolism. In our experience, a strong dilution is favorable for the survival of sperm cells during cold storage, provided that a suitable diluent with a physiological osmolality is used. With a modified (see below) version of a diluent described in [28], motility was maintained at virtually "unlimited" ($>10^6$ times) dilution (Woelders, not published). Sexton [29] reported a negative effect of semen dilution, although Blesbois and de Reviers [30] explained how in fact specific fractions of seminal plasma were responsible for the declined fertility in diluted semen. A positive effect of dilution of chicken semen was reported by Parker and McDaniel [31]. The latter authors also showed how the effect of dilution depends strongly on the type of diluent used, with negative effects of diluting with seminal plasma and positive effects of diluting (up to 200-fold dilution rate) with culture medium MEM. For insemination of chickens with "fresh" semen, one could easily dilute the semen 40 times to provide a sufficient (*see* [19]) sperm dosage in an acceptable insemination volume. We would advise to use a dilution rate of >10 times if cold storage, e.g., during 24 h or 48 h, is required. It has been clearly demonstrated that good fertility can be obtained with stored poultry and turkey semen [25–28]. Also for freezing of avian semen, higher dilution rates appear beneficial [22], and we have

seen in chicken (unpublished) and have reported for turkey semen [32] that freezing at a fixed concentration is better than at a fixed dilution rate.

A diluent that is a good extender for cold storage of "fresh" semen may also likely be a good base medium for freezing semen, assuming that "happy sperm are freezable sperm". Obviously, a diluent that causes cellular stress by, for example, inappropriate pH, osmolality, electrolyte composition and concentration, etc. provides a wrong starting point for cells to cope with the additional stresses of freezing and thawing. Electrolyte concentration seems also important, as solutes become strongly concentrated during freezing. Thus, a freezing medium may consist of a good fresh extender supplemented with cryoprotective agents (CPAs) such as glycerol, etc. and possibly other components that are deemed beneficial. Original extenders for poultry semen were "inspired" by the composition of seminal plasma [33, 34] in that they contain a high concentration glutamate, serving as an organic anion. A freezing semen that is often used is Lake's "Solution 1" [35] (*see*, for example, [32] and references therein). This medium as well as the fresh extender "A" [34] contained very little pH buffering capacity. Addition of buffering substances (phosphate and sulfonic acid-based, zwitterionic "Good's" buffers like MES, BES, TES, and Hepes) was then introduced [28, 29, 36]. Lake and Ravie [28] reported best fertility with 24-h cold-stored semen when using an MES (pH 6.8) or BES (pH 7.1) buffered diluent, compared with similar diluents but with other buffers and other pH values.

In our own experience, we also obtained very good results when using the BES-containing Lake and Ravie [28] diluent of pH 7.1 ("L&R 7.1") as fresh extender or as "base" for a freezing medium, compared with a large number of other possible poultry semen extenders, culture media, or mammalian semen extenders we tried for poultry semen. However, the osmolality of the L&R 7.1 medium (411 mOsm/kg of water) is outside the physiological range. At low dilution rates (e.g., 1:1 [28]), this poses no problem, as the final osmolality remains at an acceptable value between that of seminal plasma and that of the diluent. At higher dilution rates, the resulting dehydration of the sperm cells inhibits sperm motility (cf. [37]). We therefore modified the L&R 7.1 medium to have an osmolality of 325 mOsm/kg of water, similar to that of poultry semen (referred to as "ASG poultry extender" in [21, 32]). We have used this medium since 2004 as a fresh extender and as base freezing medium, combined with glycerol, dimethylacetamide (DMA), or other cryoprotectants. We obtained better fertility with frozen/thawed poultry [21] and turkey [32] semen frozen in "ASG"-based freezing medium compared with Lake's freezing medium ("Solution 1″ [35]). Thanks to the physiological osmolality, the ASG extender can be used as fresh extender at higher

dilution rates (e.g., 10× diluted), providing excellent longevity of cold-stored poultry semen (Woelders, in preparation). We have successfully applied the ASG poultry extender also as fresh extender and as freezing medium base for semen of cranes, tragopan pheasants, ducks, and geese, yielding better fresh longevity and better post-thaw motility compared with commercial Avidiluent extender (IMV Technologies, L'Aigle, France) (Woelders and Zuidberg, unpublished). Therefore, the ASG extender (modified L&R 7.1) is our preferred medium for avian semen.

1.4 Cryoprotective Agents

Glycerol is seen as a good CPA for poultry semen, but it is contraceptive if present together with the semen in the hen. While the mechanism of the contraceptive effect isn't fully elucidated, research with in vitro tissue cultures indicated that glycerol and tissues of the hen genital tract have an interaction that negatively affects sperm viability [38]. The contraceptive effect of glycerol is not seen in mammalian species. Methods have been devised to remove glycerol from poultry semen. Also, other CPAs that do not appear to have the contraceptive effect have been tried as alternatives to glycerol. DMA (0.6–0.7 M) and glycerol (1.1–1.2 M) both appear effective CPAs, as high post-thaw fertility rates of ±90% have been reported for DMA [20, 39–41] and for glycerol [19, 22, 42]. However, Blesbois and coworkers reported glycerol to be the more effective than DMA with low fertility lines [24]. Also with DMSO (0.6 M), fertility around 90% was reported [43].

As pointed out by Donoghue and Wishart [44], there are many confounding factors affecting comparisons of CPAs between or within studies, such as the use of different CPA concentrations, freezing rates, freezing methods, and "base" diluents. A rationale for testing CPAs at different cooling rates may be that they may have different optimal cooling rates when compared under otherwise identical conditions. For instance, in split sample comparison of glycerol and DMA at equal concentrations and equal cooling rates, we observed similar post-thaw sperm survival in poultry semen frozen at cooling rates between 4 and 200 °C/min, but glycerol was not effective at cooling rates above 200 °C/min, at which DMA was more effective than glycerol [21]. Also, comparisons of CPAs may be made at different CPA concentrations, as the apparent tolerated concentrations during freezing and thawing may differ for different CPAs. Toxicity of the CPAs tested in semen without freezing/thawing may differ from the toxicity (or effectiveness) of these compounds during freezing and thawing. For instance, Lake and Ravie [27] showed that fertility rate of fresh semen with DMA or propanediol (PD) was close to control (no CPA), whereas fertility of frozen semen was much lower when using PD compared with either DMA or glycerol. Comparing fertility of semen frozen with glycerol or with alternative CPAs,

Chalah and coworkers [40] obtained fertility with semen frozen with DMA similar to that obtained with fresh semen, while fertility with glycerol and dimethylformamide (DMF) was a bit lower. Also, Tselutin and coworkers [20] reported higher fertility with DMA than with glycerol, although this depended on the method (cooling rate) used. Semen frozen by plunging droplets of semen with DMA in LN_2 (pellet method) resulted in very good fertility, but not semen frozen conventionally in straws at much lower cooling rates [20, 22]. Woelders and coworkers [21] showed that very good fertility can be equally obtained using DMA as CPA with either pellet and straw freezing, provided that also the straws are frozen at a very high cooling rate (200 °C/min). Thélie and coworkers [22] found better fertility with glycerol than with DMA but using DMA in a straw method (i.e., different from the pellet method in [20, 40]) and using a lower cooling rate than that used in [21].

All in all, good fertility of ±90% can apparently be obtained with frozen-thawed poultry semen using either DMA or glycerol as cryoprotectant. However, when using glycerol, the semen cannot be inseminated right from the straw. Instead, extra steps are needed after thawing to remove glycerol prior to insemination, which implies extra labor and costs. Methods to remove glycerol include dialysis [2, 45], stepwise dilution, followed by centrifugation [22, 42], and discontinuous density gradient centrifugation [46].

1.5 Other Stabilizing Additives

For semen of mammalian species, the presence in the freezing medium of milk components or egg yolk is beneficial, if not essential, for post-thaw sperm survival and fertility. However, it seems there is little if any evidence for positive effects of yolk or milk components for avian semen. Instead, chicken egg yolk reduces fertility of chicken semen, but not of turkey and red jungle fowl [47–49]. Low-density lipoprotein extracted from egg yolk [50], egg yolk plasma, and soybean lecithin [51] were found beneficial for freezing poultry semen.

The use of sugars and other non-permeating neutral (nonelectrolyte) solutes in (cryo)preservation media results in a lower ionic strength, which may be beneficial [37, 41], especially as salts and other solutes become strongly concentrated in the "unfrozen fraction" during freezing. Very high ionic strengths affect lipid membranes and (membrane) proteins and may lead to "salt loading" of the cells. Sugars and other neutral solutes are sometimes referred to as "non-permeating cryoprotectants." However, in media with normal (physiological) osmotic strength, the ratio of electrolytes/nonelectrolytes will not influence freezing point depression, or the degree of shrinking of the unfrozen fraction and of the cells therein during ice formation, nor can it affect intracellular cryobiological properties. High osmolalities (be it by sugars or any other solutes) can work to reduce the chance of intracellular ice formation and

allow higher cooling rates [37]. In fact, this is how sugars were used (but without real success) before the discovery of the cryoprotective properties of permeating CPAs [52] (cf. [2]). The higher osmolalities are already a potential cause of cell stress and damage even before freezing has started. Therefore, the term non-permeating CPA may not be appropriate, notwithstanding the fact that ionic strength and overall medium composition can be an important factor for cryosurvival.

A similar discussion is valid with regard to macromolecules such as polyvinylpyrrolidone [35], BSA, etc. Unless used at very high concentrations, these compounds are not likely to affect cryobiological properties of the medium, but these compounds may have specific interactions with the cell surface that may be beneficial during or after freezing and thawing. For instance, the presence of BSA after thawing can improve the apparent sperm motility in poultry semen.

Another potential class of additives are the antioxidants. There is much recent literature on the effect of antioxidants in the freezing medium on post-thaw semen quality of avian semen. Additives with antioxidant activity investigated in avian semen include catalase, superoxide dismutase (reduced) glutathione, vitamins E and C, selenium, carnitine, hyaluronic acid, lycopene, oleic acid, linoleic acid, coenzyme Q10, resveratrol, and quercetin (*see*, for example, [53–55] and references therein to earlier studies in both avian and mammalian species).

Unlike bull semen extenders, poultry extenders described in the literature often do not contain antibiotics, although antibiotics may be added without negatively affecting semen fertility [56]. For cold storage of "fresh" semen, the presence of antibiotics may improve longevity.

1.6 Freezing Methods

Avian semen is generally frozen in 0.25-mL straws or in "pellets." Frozen pellets can be obtained by dropping approximately 50 μL droplets straight onto the surface of liquid nitrogen (LN_2) [39]. While this can yield very good results, the packaging in straws is often preferred as it allows easier labelling of the semen. Another disadvantage of pellet freezing is that a special device is needed for rapid thawing of the pellets.

Cooling rates used may differ according to species and also seem to be different for different types of CPA used. In addition, for a given species and CPA, different authors may use or advise different cooling rates. The pellet freezing method but also studies on freezing in straws [21] indicate that poultry spermatozoa can tolerate (and may perhaps prefer) quite high cooling rates. For instance, the average cooling rate of 50-μL droplets in LN_2 is approximately 600 °C/min (it takes approximately 20 s to cool and freeze from +5 °C to −196 °C).

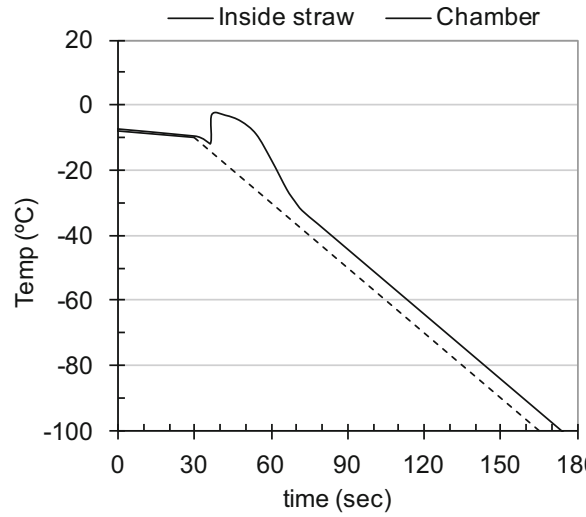

Fig. 1 Schematic representation of time course of temperature inside straws filled with semen in a programmable (controlled rate) freezer. Ice nucleation occurs at a temperature below −10 °C. After ice nucleation occurs, the liberation of heat of fusion causes a deviation of the cooling rate inside the straw from the programmed cooling rate of the freezing chamber

Freezing in straws can be done with a programmable (controlled rate) freezer, or with not controlled rate methods. It is often stated that "controlled rate" freezers would be better, as they allow better control over the freezing rate. This is a bit misleading. In fact, the cooling rate inside the semen during the critical phase of the freezing process, i.e., after ice nucleation has occurred and during the rapid growth of extracellular ice masses, deviates from the programmed temperature time course of the freezing chamber due to the generation of latent heat of fusion. This is schematically shown in Fig. 1, in which the temperature inside the straw after ice nucleation first remains close to the freezing point of the freezing medium followed by a brief period in which the cooling is steeper than programmed, as the straws "catch up" with the programmed cooling curve.

Not controlled rate freezing methods do not allow to incorporate a gentle slow cooling phase before ice nucleation. In fact, the cooling rates prior to ice nucleation and after dissipation of heat of fusion, respectively, are similar (Fig. 2). In contrast, programmable freezers can accommodate a first ramp of slow cooling to the nucleation temperature (Fig. 1). A second feature of not controlled methods is that at lower temperatures, as the straw temperature comes close to that of the coolant, the freezing rate declines. This is not necessarily a disadvantage. Woelders and Chaveiro [57] argued on the basis of theoretical considerations that this may actually be an advantage. In practical work, there is no evidence that the two

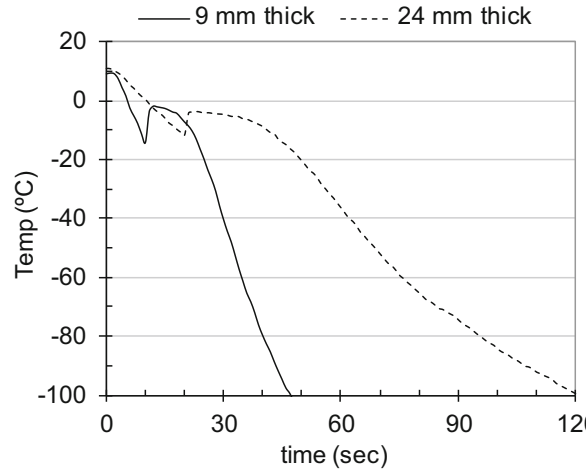

Fig. 2 Freezing curves recorded in 0.25-mL straws placed on a "Styrofoam rack" floating on a surface of LN_2. The overall cooling rate can be set by choosing the appropriate Styrofoam thickness (see this figure). This method results in reproducible sigmoidal freezing curves yielding good freezing results for avian semen

mentioned features of not controlled methods pose a threat to post-thaw function of avian semen, compared with programmable freezers.

Examples of nonprogrammable freezing methods are, for instance, the abovementioned "pellet method" and freezing in static LN_2 vapor. Freezing straws in vapor can be done by placing the straws on a typical (metal) straw rack in an insulated box containing a level of LN_2. The cooling rate can be varied by varying the distance of the straws to the surface of LN_2. This method has a poor reproducibility as the boiling of LN_2 caused by the heat of the metal rack causes turbulence, disturbing the built-up layer of LN_2 vapor. However, reproducibility can be very good when placing the straws on a Styrofoam raft, which is then placed floating on the surface of LN_2, as proposed by Dong [58]. We have modified the "raft" concept by making Styrofoam frames, which means that the straws are exposed to the nitrogen vapor over almost their complete length, while both ends of the straws rest on opposing sides of the frame. This gives a very repeatable relation between the thickness of the frame (i.e., the distance between the straws and the LN_2 surface) and the cooling rate. Cut out grooves are provided in the sides of the frames to allow easy and regularly interspaced placing of the straws using a "distribution block" for mini straws (IMV Technologies, L'Aigle, France). This freezing method results in highly repeatable sigmoidal freezing curves [32], with moderate cooling rates during the dissipation of heat of fusion followed by high cooling rates after the dissipation of heat of fusion (cf. [57]. The overall cooling rate can be set by choosing the appropriate

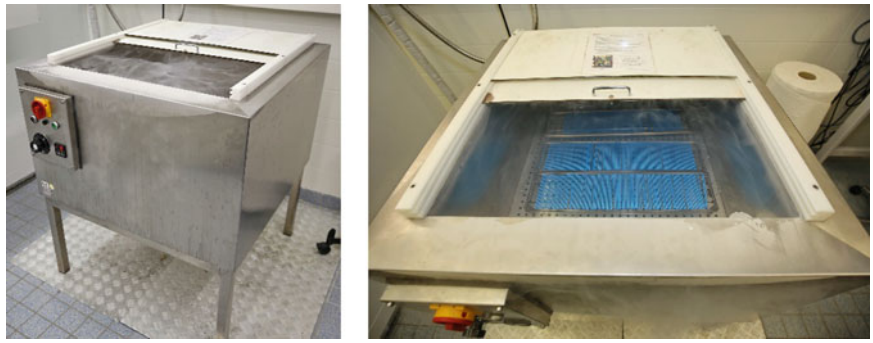

Fig. 3 Custom-built ventilated nitrogen vapor freezer, with a constant vapor temperature. These machines are robust and reproducible and easy to work with, providing very easy access for placing the racks and for removing the straws. They give reproducible sigmoidal freezing curves (similar to the curves shown in Fig. 2). The cooling rate depends on the set freezing chamber temperature and the ventilation rate (windchill) in the freezing chamber

Styrofoam thickness (Fig. 2). This method resulted in good results for freezing poultry (Woelders et al., unpublished) and turkey [32] semen.

A similar method, suitable for high-throughput freezing of larger batches of straws, uses ventilated LN_2 vapor in a large cabinet at a constant vapor temperature. The cooling rate is determined by the vapor temperature and the ventilation rate ("windchill factor"). Several 70-straw or 100-straw racks can be accommodated and frozen simultaneously with little effort. The CGN animal genetic resource (AnGR) bank is equipped with several of such custom-built devices to freeze mammalian and avian semen (Fig. 3). This freezing method also provides highly repeatable sigmoidal freezing curves, similar to that obtained with the static vapor method with the Styrofoam raft method (cf. Fig. 2).

2 Materials

2.1 General Laboratory Materials and Equipment

1. Semen collection tubes.
2. Thermostatic cold box (4–6 °C).
3. Tubes, tube racks.
4. Open top cooler cabinet or cold room (4–6 °C).
5. Semen sperm concentration apparatus/photometer (IMV, L'Aigle, France, or similar).
6. (Fluorescence) microscope with phase contrast.
7. Dewar.
8. LN_2.
9. LN_2 storage tank, with canisters, goblets, etc.

Table 1
Medium composition of "ASG-PE" (= modification of medium "7.1." from [28])

	g/100 mL	mmol/L
Sodium-L-glutamate.H_2O	1.21	64.7
Tri-potassium-citrate.H_2O	0.102	3.14
Magnesium acetate.$4H_2O$	0.064	2.97
D-(+)-glucose monohydrate	0.53	26.5
BES (*N,N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid)	2.43	114
NaOH	0.185	46.2
Pure water (e.g., Milli-Q)	To 100 mL	
pH = 7.1		

10. Tweezers, gloves.
11. 0.25-mL straws (IMV, L'Aigle, France, or similar).
12. Heat sealer for straws, or use PVA powder.
13. Freezing equipment (static LN_2 vapor, ventilated LN_2 vapor, or programmable).
14. Thermocouple equipment (thinnest possible copper-constantan Type K thermocouple wires with computer interface, Omega, Norwalk, CT).

2.2 Diluents

1. "ASG-PE": medium composition of "ASG-PE" (= modified medium "7.1.," Lake and Ravie, 1979) is prepared as described in Table 1.
2. "Solution 1": medium composition of "Solution 1" [59] is prepared as described in Table 2.

2.3 Freezing Media

1. Freezing can be done with dimethylacetamide (DMA) or glycerol as CPA (*see* **Note 1**). DMA is described at 6% (~60 g/L) [39] or at 0.6 M [21]. The final concentration of the CPA can be obtained by adding one volume of three times concentrated CPA solution in diluent to two volumes prediluted semen. Table 3 shows how to prepare three times concentrated CPA medium (*see also* **Note 2**).

3 Methods

3.1 Semen Collection and Processing

1. Semen is collected by the abdominal massage technique [60] (*see* **Notes 3** and **4**).

Table 2
Medium composition of "Solution 1" [59]

	g/100 mL	mmol/L
Sodium-L-glutamate.H_2O	1.92	102.6
Magnesium acetate.$4H_2O$	0.08	3.73
Fructose	0.8	44.4
Potassium acetate	0.5	50.9
Polyvinylpyrrolidone	0.3	0.08
Pure water (e.g., Milli-Q)	To 100 mL	
pH = 6.9		

Table 3
Preparation of "three times concentrated" CPA medium. This medium (cooled) can be added as one volume to two volumes of prediluted cooled semen to obtain the desired final CPA concentrations (*see* Notes 1 and 2)

	3× concentrated		Final concentration	
	g/100 mL	Mol/L	g/100 mL	Mol/L
Weigh all chemicals for the diluent as given in Tables 1 or 2 Dissolve in pure water (e.g., Milli-Q) Also add 15.68 g DMA	15.7	1.8	5.2	**0.6**
Or Also add 33 g glycerol Fill with pure water to 100 mL	33	3.6	11	**1.2**

2. A special glass graduated funnel-shaped tube may be used for sperm collection or just a standard graduated tube. Preferably, the semen extender (ASG poultry extender (ASG-PE)) and the collection tubes are placed in a 25 °C incubator in the barn. This is only necessary at low barn temperatures.
3. A cock is fixed by one person between the arm and body with the tail pointing forward, holding the legs between the fingers, while a second person collects the semen. This person strokes a few times with his hand over the back of the cock from shoulders toward the tail, exerting some pressure. This will cause the tail to be raised. The behavior of the animal indicates its readiness to ejaculate, and the semen collector will hold the prewarmed tube underneath the cloaca while pinching the cloaca with two or three fingers. Try to collect the semen without feces or other contaminations. It is advised to withdraw feed prior to planned semen collection.

4. The chicken ejaculate may have a volume of approximately 0.5 mL and may contain 2–6 $\times$ 10^9 spermatozoa per mL (*see* **Notes 5** and **6**).
5. The tube is closed with a stopper to prevent evaporation (dehydration) and is placed in a thermostat-controlled cool box at 5 °C (*see* **Note 7**).
6. The semen is preferably cooled relatively rapidly (but not abruptly), starting cooling directly after collection and predilution with extender. Cooling can be done, for example, by placing the tubes of pre-extended semen in a 5 °C cool box. The heat capacity of the tube and the volume of semen will result a slow enough cooling (*see* **Note 8**).
7. When ejaculates from all cocks are collected, the semen is transported to the laboratory for further processing and freezing. All further handling is performed at 5 °C (cold room or open top cooler cabinet).

3.2 Quality Assessment

1. The sperm concentration can be determined using a photometer, which measures transmission decrease resulting from light scattering by the sperm cells. This requires a very small volume of the ejaculate which is diluted with a saline. Each species would require a dedicated calibration, preferably made at various sperm concentrations as the extinction is not exactly proportional to sperm concentration. Calibrations can be done by using a hemocytometer with immobilized sperm cells.
2. Motility can be estimated in extended semen, e.g., using a tenfold or stronger dilution (with an appropriate diluent such as ASG-PE). Use a final sperm concentration that would allow to see individual freely swimming sperm cells with a limited (10–100) number of cells per field of view (*see* **Note 9**). Four μL of the diluted semen preparation can be placed on a microscope slide and covered with a coverslip. The percentage of motile sperm is estimated using phase contrast microscopy in five different fields of view at different places of the coverslip, and the estimates are then averaged (*see* **Note 10**).
3. Plasma membrane integrity can be estimated in (strongly) diluted semen. Add a volume of poultry extender containing DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride), having the same temperature as the semen, to reach a final DAPI concentration of 5 μM. Add just a smidge of 0.5% glutaraldehyde solution (e.g., 10 μL added to 200 μL diluted DAPI-stained semen) to immobilize the live sperm cells and place the tube at RT for 4 min (*see* **Note 11**). Then place 3 μL on a slide covered with an 18 $\times$ 18 mm coverslip, and assess a minimum of 200 cells in different fields of view in a microscope combining phase contrast and fluorescence microscopy.

4. If available, one may employ computer-assisted sperm analysis (CASA) for motility and other variables, or flow cytometry, using fluorescent dyes for plasma membrane integrity, acrosome intactness, or mitochondrial function.

3.3 Fresh Storage

1. The semen is best stored at a constant temperature of approximately 5 °C. In routine practice, the dilution rate for fresh poultry semen is usually very low, but a higher dilution rate (with an extender of the appropriate osmolality) would be beneficial for longevity during cold storage. A tenfold dilution of the semen with ASG-PE would still be compatible with a sufficient sperm number for insemination with fresh semen (*see* **Note 12**).

3.4 Semen Freezing

A relatively high sperm dosage is required when using frozen-thawed semen [19]. In chicken hens, an inseminate volume of 0.2 mL is possible without having too much backflow. A sperm concentration of 1200 × 106 sperm cells/mL would thus give 240 million total sperm per dose. This final sperm concentration is usually feasible in chicken, where initial ejaculate sperm cell concentrations of 4000 million per mL or more can be expected, but this may be different in special cases. If glycerol is used as CPA, one may actually freeze at lower sperm concentrations, as the semen is reconcentrated by centrifugation in the course of post-thaw glycerol removal. In order to compare freezing methods and to validate a chosen freezing method as to the actual cooling rate obtained inside the straws, it is advised to measure cooling rate using thin "Type K" copper constantan thermocouples mounted inside "dummy" straws. A tiny hole can be bored in the side of an empty straw, and a thermocouple wire can be placed with the sensing "tip" inside the straw and fixed with a bit of glue. After the glue is cured, the straw can be filled with freezing medium and sealed in the normal fashion. The thermocouple is connected to the computer interface, and the dummy straw is placed between straws to be frozen.

3.4.1 Method DMA in Straws

With this method, the semen can be used directly for insemination after thawing. No need to remove the cryoprotectant prior to insemination. Use ASG-PE as the extender. The sperm concentration of the prediluted semen is determined with a spectrophotometer or hemocytometer. Handling is performed at 5 °C (cold room or open top cooler cabinet).

1. The semen can then be further diluted with ASG-PE to a chosen sperm concentration (e.g., 1.8 × 109 sperm/mL). 0.25-mL straws are printed with a straw printer.
2. Then, half a volume of ASG-PE with three times the final concentration of DMA, e.g., 1.8 mol/L, is added to 1 volume

of semen. Final concentrations will thus be 1200 million sperm cells/mL and 0.6 mol/L DMA.

3. The semen may be held in the DMA medium at 5 °C, if wanted, for any time up to 1 h. Straws are filled and sealed. Make sure that the straw contains a 10-mm-long air pocket. This is especially important at the relatively high cooling rates employed.
4. Sealing can be done by pressing the (wet) open end of the straw on polyvinyl alcohol (PVA) powder, which then forms a plug. But it is to be preferred to make a heat seal instead, e.g., with a straw filling machine.
5. The straws are then frozen. Use a freezing rate of 200 °C/min. This is the rate as measured inside the straw after the dissipation of heat of fusion, e.g., between −10 and −60 °C. Programmable freezers are not intended for such high cooling rates. One may use a programmable freezer, but set at a constant temperature of, for example, −160 °C (*see* **Note 13**). Straws are placed on a steel rack for freezing 0.25-mL straws (IMV, L'Aigle, France). Depending on the ventilation rate and the strength of the jets of injected LN_2, it may be necessary in these programmable freezers to restrain the straws, which can be conveniently done with a rubber band stretching along the length of the rack. The rack with straws is then placed inside the precooled freeing cabinet. After 2 min, the straws can be removed from the cabinet and be plunged in LN_2.
6. CGN is using custom-built ventilated nitrogen vapor freezers, set at a constant vapor temperature of −140 °C (Fig. 3). This works similarly as the freezing cabinet described above, except that these machines allow very easy access to place the racks and for removing the straws and do not have the risk of blowing away the straws from the racks. Figure 3 shows two images of this apparatus.
7. A simple low-cost way is to place the straws on a Styrofoam "floating rack," as described above, with a thickness of 12.5 mm. A large (approximately 30 × 30 cm) Styrofoam box filled with a layer of LN_2 is prepared. The rack with straws is then placed floating on the surface of the LN_2. This will provide the desired cooling rate. After 1 min, the straws can be plunged in LN_2.
8. A last alternative is to use a programmable freezer and freeze with a constant rate of 50 °C/min (= maximum rate of most programmable freezers).
9. Straws are thawed in a 5 °C water bath, and the thawed semen is best maintained at 5 °C until use for semen assessment or insemination as it will deteriorate faster at elevated temperatures (*see* **Note 14**). Straws are taken from liquid nitrogen and

submersed in the 5 °C water while being moved vigorously through the water during 30 s. This is to maximize warming rate and prevent growth of an insulating sheath of ice outside the straw. Despite the low temperature of the water bath, the thawing rate is still high enough (average thawing rate between ± −190 and + 5 °C is 500–600 °C/min). Do not thaw bundles of straws as this will reduce the thawing rate.

10. For insemination, the semen can be used as is. The semen does not need to be warmed first. For post-thaw semen assessment, the thawed semen must be extended with ASG-PE of 5 °C to the appropriate low concentrations needed for the chosen sperm assessment method. It is advised to do the initial post-thaw dilutions in a stepwise fashion.

3.4.2 Method DMA in Pellets

Semen is prepared exactly as described in the "Method DMA in Straws" above. Tselutin [39] describes that the semen is frozen 1 min after adding DMA, although longer exposure to DMA prior to freezing does not appear to be harmful (Woelders et al., in preparation).

1. Prepare a dewar with LN_2.
2. A "blue" tip of a 1-mL pipette is cut off just a bit such that droplets formed are approximately 0.5 mL.
3. Then the semen is pipetted with this tip, and droplets are dropped onto the surface of the LN_2. It is best to wait for the droplet to be cooled (20 s) and then sinks, or else droplets will collate together which will affect cooling and thawing rates.
4. A sort of "tea sieve"-like insert may be convenient for easy collection of the pellets for storage.
5. The pellets can be conveniently thawed using a custom-built apparatus as shown in [39]. The pellets are thawed by contact to a warm (60 °C) surface without the risk of overheating the semen, as the semen drips down as soon as the pellet melts.
6. The collected semen can be maintained at 5 °C.

3.4.3 Method Glycerol in Straws

1. Semen is prepared as described in the "Method DMA in Straws" above, using either ASG-PE or Lake's "Solution 1" and using glycerol as CPA. Glycerol is not suitable for very high cooling rates as obtained in the pellet method. Also, the relatively high CPA concentration of 1.2 M allows freezing at lower cooling rates. Cooling rates between 4 and 50 °C/min seem appropriate. Thélie and coworkers [22] used 7 °C/min with good post-thaw fertility results. That means that straws can be frozen in a controlled rate freezer. Alternatively, straws can be frozen in static nitrogen vapor, as described above in "Method DMA in Straws," but using Styrofoam floating racks of 5-cm thickness.

2. When the freezing program is complete (e.g., reaching −100 °C or lower), plunge straws into LN_2 and store.
3. Straws can be thawed exactly as described above in "Method DMA in Straws." If glycerol is used as CPA, the glycerol must be removed, or its concentration substantially lowered before insemination. A method that is practical and simple, and can yield apparently good results [22, 59], is dilution in extender and reconcentration of the sperm cells by centrifugation. Semen is progressively diluted in ASG-PE in a stepwise fashion [42] with gentle agitation to a final dilution of minimally ten times. The equilibration of glycerol (at 5 °C) is probably very fast, but one may use 2 min intervals between dilution steps. The diluted semen is then centrifuged at 500 × *g* at 4 °C [22]. The sperm pellet is then gently resuspended in the appropriate volume of ASG-PE.

4 Notes

1. Glycerol must be removed prior to insemination, for example, by stepwise dilution, followed by centrifugation [22, 42].
2. This procedure (Table 3) of accommodating the CPA as well as all solutes as used in the medium without CPA into the same final volume as used in the medium without CPA is different from adding CPA to a ready isotonic medium. If the latter would be done, the molar concentration of non-permeating solutes would be lowered by the addition of CPA, causing swelling of the cells above their isotonic volume after equilibrating with the CPA.
3. The massage technique [60] is also applied (in adapted form) to nondomestic species, but see refs. 15, 16 for species specific recommendations.
4. Semen may be collected twice or three times per week. Training the males for collection and regular collection make subsequent collections easier. More importantly, regular collection results in better semen quality.
5. Ejaculate volume and sperm concentration depend on breed and animal, and different values may apply to other species than chicken.
6. The ejaculate is quite viscous. Directly after collection, the volume is roughly estimated, and an equal volume of ASG-PE is added to wash down the semen and supply an initial predilution. Semen with inadequate volume or too watery or contaminated with blood or feces must be discarded.
7. For some purposes, pooled ejaculates may be collected from a series of cocks. One method often employed for practical reasons is to place a few mL of extender in a collection tube and

then collect semen of a number of roosters consecutively in that collection tube. Alternatively, one may collect semen from roosters consecutively in a collection tube and after each rooster wash down the ejaculate with 0.5 mL of extender.

8. If small tubes and/or small volumes are used, one may place the tubes surrounded with tissue paper in a beaker or tube rack to prevent a perhaps too abrupt cooling.
9. In frozen semen, the apparent sperm motility may be better if the final dilution contains approximately 10 mg of bovine serum albumin per mL.
10. Poultry semen motility can best be estimated at room temperature rather than at 40 °C, as the sperm cells may be too agile on a heated stage.
11. The fixative (cross-linking agent) glutaraldehyde can render cell membranes permeable for salts. However, it does not make the spermatozoa permeable to DAPI (*see*, for example, [61]).
12. During prolonged storage, e.g., several days, bacterial growth may play a role especially if no antibiotics are used in the extender (but, *see* [56]).
13. The actual cooling rate will depend on the freezing cabinet temperature and the ventilation rate (windchill) in the freezing cabinet.
14. One may use a thermostatic cooled bath. An easy low-cost alternative is to have a Styrofoam box filled with water and another one containing ice flakes. Simply check water temperature regularly, and add a handful of ice flakes if temperature rises above 7 °C.

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