

Petitioner ABS Global, Inc. - Ex. 1030, p. 1

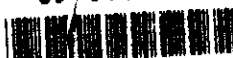
PATENT APPLICATION



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INTERFERENCE SEARCHED			
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Petitioner ABS Global, Inc. - Ex. 1030, p. 3

ISSUE SLIP STAPLE AREA (for additional cross references)

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FEE DETERMINATION	MM	2891	5/31
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FORMALITY REVIEW	A.M	56 580	07-19-00
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INDEX OF CLAIMS

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PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

08/09/2000 NVILLARI 00000017 501189 09577246
01 FC:201 345.00 CH

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09/577,246	05/24/2000	514	1651	22091-701 DI

APPLICANT
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ADDRESS
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TITLE
METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

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ABSTRACT OF THE INVENTION

The present invention provides a method of cryopreserving sperm that have been selected for a specific characteristic. In a preferred embodiment, the method is employed to freeze sex-selected sperm. Although the cryopreservation method of the invention can be used to freeze sperm selected by any number of selection methods, selection using flow cytometry is preferred. The present invention also provides a frozen sperm sample that has been selected for a particular characteristic, such as sex-type. In preferred embodiments, the frozen sperm sample includes mammalian sperm, such as, for example, human, bovine, equine, porcine, ovine, elk, or bison sperm. The frozen selected sperm sample can be used in a variety of applications. In particular, the sample can be thawed and used for fertilization. Accordingly, the invention also includes a method of using the frozen selected sperm sample for artificial insemination or in vitro fertilization.

**APPLICATION
FOR
UNITED STATES PATENT
on**

METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

by

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Docket No.: 22091-701 DIV2

METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

A
FIG 5
AI
The application claims the benefit of U.S. Provisional Application No. 60/167,423, filed November 24, 1999.

FIELD OF THE INVENTION

The invention relates to a method for freezing sperm selected for a particular characteristic, as well as to a frozen selected sperm sample and methods of using such a sample.

10 The invention is particularly useful for preserving sex-selected sperm.

BACKGROUND OF THE INVENTION

Over half a century ago, artificial insemination was introduced in the United States as a commercial breeding tool for a variety of mammalian species. Although artificial insemination was initially limited to regions relatively close to the site of sperm collection, advances in the cryopreservation and storage of sperm have facilitated widespread distribution and commercialization of sperm intended for artificial insemination or in vitro fertilization.

15 Further improvements in mammalian sperm collection, selection, cryopreservation, storage, and handling techniques have enhanced the ability of breeders to produce animals having desired traits. For example, advances in selection of mammalian sperm based on slight differences in physical characteristics has made it possible to separate sperm based on sex-type, that is, to select for cells containing either the X or Y chromosome. This technique allows the breeder to manipulate the relative percentage of X- or Y-type sperm in a sample and thereby determine offspring sex. The ability to select sperm based on sex-type or any other desirable characteristic provides an important tool for accelerating genetic progress, increasing production efficiency, and achieving greater flexibility in livestock management. Full exploitation of this tool, however, depends on the ability to freeze and store selected sperm.

25 A variety of methods are available for selecting cells; however, the selection and subsequent processing of sperm presents unique challenges because sperm are incapable of DNA repair and because of sperm morphology. Each sperm has an acrosome overlying the head and a tail, which are important for fertility and which are relatively susceptible to physical injury. In

addition, sperm fertility decreases with increasing time between collection and use. As most of the available selection methods involve physical stresses and take time, selected sperm are typically somewhat compromised compared to non-selected cells. Fertility may be further reduced if the selection technique involves significant dilution. It has been suggested that this
5 “dilution effect” may be due to the loss of protective components in seminal plasma.

Flow cytometry is a particularly efficient selection method that has been employed for sorting sperm by sex-type. However, sorted sperm are subject to stresses beyond those normally encountered in standard artificial insemination or in vitro fertilization protocols. In particular, flow cytometry is time consuming, and, because of the physical constraints of flow cytometers,
10 sperm must be diluted for sorting to levels that are not optimal for storage (usually to on the order of 10^5 - 10^6 /ml). Furthermore, sorted sperm intended for artificial insemination must be concentrated so that conventional packaging and delivery equipment can be used. The need for a concentration step thus exposes already somewhat compromised sperm to additional physical stresses.

15 The freezing of sperm also invariably reduces fertility, motility, and/or viability, and, although techniques for freezing unselected sperm are well known, no technique for cryopreservation of selected sperm has been described.

SUMMARY OF THE INVENTION

20 The present invention provides a method of cryopreserving sperm that have been selected for a specific characteristic. The method is particularly useful for cryopreserving sperm selected by a method that results in dilution of the sperm, since the method provides for the isolation of sperm from a selected sperm sample, followed by addition of a final extender to the isolated sperm to produce a suspension having a desired concentration of sperm. In a preferred
25 embodiment, the method is employed to freeze sex-selected sperm. Although the cryopreservation method of the invention can be used to freeze sperm selected by any number of selection methods, selection using flow cytometry is preferred.

The present invention also provides a frozen sperm sample that has been selected for a particular characteristic, such as sex-type. In preferred embodiments, the frozen sperm sample
30 includes mammalian sperm, such as, for example, human, bovine, equine, porcine, ovine, elk, or

bison sperm. Also within the scope of the invention is a container including a frozen sperm sample according to the invention.

The frozen selected sperm sample can be used in a variety of applications. In particular, the sample can be thawed and used for fertilization. Accordingly, the invention also includes a method of using the frozen selected sperm sample for artificial insemination or in vitro fertilization.

DETAILED DESCRIPTION OF THE INVENTION

The present invention allows cryopreservation of sperm that have been selected for a particular characteristic, facilitating storage and/or shipment of selected sperm samples to sites distant from the collection site. Thawing yields viable sperm that can be used in procedures such as artificial insemination (“AI”) and in vitro fertilization (“IVF”). This result was surprising because of the well-documented fragility of sperm. Prior researchers had demonstrated that the stresses associated with various selection methods or with cryopreservation resulted in significant losses in fertility and/or viability. The present inventors have demonstrated, for the first time, that pregnancies can be achieved with sperm that have been selected and then frozen.

The invention represents an important advance in livestock management, where selection of sperm for use in such procedures can be used to increase the production of offspring having desirable traits. For example, selection to obtain sperm carrying either the X or the Y chromosome allows control over offspring sex, which is advantageous for producers of animals such as dairy or beef cattle. Sex selection also finds application in breeding valuable (e.g., show or race horses) or endangered animals. The ability to freeze selected sperm, which the invention provides, will enable widespread use of such selection methods to, e.g., increase livestock production efficiency as well as quality.

Definitions

The term “acrosome” or “acrosomal cap” refers to the cap that covers the anterior half of the head of sperm and that contains enzymes necessary for ovum penetration.

The term “sex-type” refers to the type of sex chromosome present in the sperm (i.e., the X or Y chromosome).

The term “capacitation” refers to the specific changes a sperm undergoes to develop the capacity to fertilize ova, such as enzymic changes on the surface of the acrosome that lead to release of acrosomal enzymes that facilitate penetration of the sperm into the ovum.

As used with reference to sperm, the term “cryoprotectant” refers to a molecule that
5 protects sperm during a freeze-thaw cycle, promoting survival and retention of fertilizing capacity.

The term “dilution effect” refers to the rapid decline in motility and/or viability of sperm when highly diluted.

As used herein, the term “selection” refers to a method whereby a sample is subdivided
10 based on presence or absence of a specific characteristic (unless context dictates otherwise). Thus, a “selected sperm sample” is a sample obtained by subjecting a source sample to selection for the specific characteristic. A selected sperm sample is therefore enriched, relative to the source sample, in sperm having the specific characteristic.

The term “sorting” is used herein to describe a selection method carried out using a
15 fluorescence-activated cell sorter (FACS).

The term “extender” refers to any medium that tends to preserve sperm viability. The term “extension” refers to the dilution of sperm with extender.

The term “initial extender” refers to a medium used to extend sperm prior to the isolation step of the method of this invention.

20 The term “final extender” refers to a medium used to extend sperm prior to the freezing step of the method of this invention.

An “organic substance” in an extender described herein is any organic substance that tends to reduce cold shock and preserve fertility of sperm.

An “energy source” in an extender described herein is any substance or substrate that
25 sperm can utilize for cell maintenance and/or motility.

The term “osmolality,” as used herein, is a measure of the osmotic pressure of dissolved solute particles in a an aqueous solution (e.g., an extender). The solute particles include both ions and non-ionized molecules. Osmolality is expressed as the concentration of osmotically active particles (i.e., osmoles) dissolved in 1 kg of water.

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Cryopreservation Method

The invention provides a method of cryopreserving selected sperm includes the following steps:

- (1) obtaining a selected sperm sample;
- 5 (2) cooling the selected sperm sample;
- (3) isolating sperm from the selected sperm sample;
- (4) adding final extender to the isolated sperm to produce a suspension of sperm; and
- (5) freezing the suspension of sperm.

10 *Obtaining a Selected Sperm Sample*

The first step in the cryopreservation method of the invention encompasses obtaining a previously selected sperm sample, as well as subjecting a source sample to any suitable selection method. Sperm from any species can be selected and frozen according to the method of the invention. The method can be carried out with sperm from domesticated animals, especially
15 livestock, as well as with sperm from wild animals (e.g., endangered species). Preferably, the selected sperm sample contains mammalian sperm. Human sperm, bovine, equine, porcine, ovine, elk, and bison sperm are particularly preferred.

Generally, the selected sperm sample contains normal, viable sperm. To this end, the ejaculate from which the sperm are obtained typically has at least about 50%, and preferably at
20 least about 75% morphologically normal sperm. In these embodiments, generally at least about 40%, and preferably at least about 60% of the sperm in the ejaculate exhibit progressive motility.

A wide variety of methods for selecting cells from a mixed populations are available, including, for example, selection based on binding of cells or cell components with antibodies, antibody fragments, or other binding partners and differential staining.

25 The invention is exemplified herein with selection based on sex-type, and sex-selected sperm for use in the invention can be obtained using any selection strategy that takes advantage of slight differences in characteristics between X- and Y-type sperm. Exemplary sex-selection methods include magnetic techniques (see, e.g., U.S. Patent No. 4,276,139), columnar techniques (see, e.g., U.S. Patent No. 5,514,537) gravimetric techniques (see, e.g., U.S. Patent
30 No. 3,894,529, reissue Patent No. 32350, U.S. Patent Nos. 4,092,229, 4,067,965, and 4,155,831). Sex-selection based on differences in electrical properties is disclosed in U.S. Patent

No. 4,083,957, and techniques that select based on differences in electrical and gravimetric properties are discussed in U.S. Patent Nos. 4,225,405, 4,698,142, and 4,749,458. U.S. Patent Nos. 4,009,260 and 4,339,434 describe selection based on differences in motility. Biochemical techniques relying on antibodies are disclosed in U.S. Patent Nos. 4,511,661, 4,999,283, 3,687,803, 4,191,749, 4,448,767, whereas U.S. Patent Nos. 5,021,244, 5,346,990, 5,439,362, and 5,660,997 describe selection based on differences in membrane proteins.

Flow cytometry is a preferred method for separating cells from mixed populations based on differential staining with fluorescent dyes or binding to fluorescently labeled molecules, such as antibodies or nucleic acids. In fluorescence activated cell sorting ("FACS"), cells are "sorted" into different populations based on the fluorescence intensity upon irradiation. FACS can be used for sex-selection of sperm because the X chromosome contains slightly more DNA than the Y chromosome. When sperm are stained with a fluorescent DNA-binding dye, X-chromosome bearing sperm absorb more dye than Y-chromosome bearing sperm and the two populations can therefore can be separated by FACS. This strategy was discussed in U.S. Patent No. 4,362,246 and significantly expanded upon in U.S. Patent No. 5,135,759 (issued to Johnson). Separation has been enhanced through the use of high-speed flow cytometers, such as the MoFlo® flow cytometer produced by Cytomation, Inc. (Ft. Collins, CO) and described in U.S. Patent Nos. 5,150,313, 5,602,039, 5,602,349, and 5,643,796, as well as in PCT Publication No. WO 96/12171.

The selection method used to obtain the selected sperm sample is preferably one that preserves sperm viability. Because of the relative fragility of sperm, normal flow cytometry techniques should generally be modified for sorting sperm. More specifically, the flow cytometry entails staining, dilution, and interrogation of cells with light. All of these steps represent stresses that can reduce sperm viability. The sensitivity of sperm to these stresses can vary between species and even between individuals within species. Such sensitivities have either been documented or can readily be determined by empirical studies, such as those described in Examples 1-5.

Modifications that enhance viability are described the patent publications discussed above. For instance, procedures that provide improved sheath and collector systems for sorting sperm are disclosed in PCT Publication No. WO 99/33956 (Application No. PCT/US98/27909). Further, Examples 1-7 below describe exemplary procedures for staining and sorting sperm.

Example 3 describes a study of the effects of laser intensity and dye concentration of post-thaw motility of sorted frozen sperm. This study indicates that the use of lower laser intensities during sorting can increase post-thaw motility.

5 The selected sperm sample can contain a variety of components besides sperm and will often contain components added to protect the sperm during the selection process. In the case of FACS, the selected sperm sample can contain component(s) of the solutions used for staining and sorting (e.g., the sheath fluid and the catch buffer).

10 In addition, the selected sperm sample typically contains an extender or extender fraction. For example, "two-step" extenders including an "A fraction" lacking glycerol and a "B fraction" containing glycerol are well known. The A fraction is added to sperm first, followed by addition of an equal volume of the B fraction. For this step, the B fraction is often divided into at least two aliquots and added sequentially; e.g., the second B fraction aliquot is added 15 minutes after the first.

15 If no extender components are present, an extender or extender fraction is typically added to the selected sperm sample before the sperm are isolated from the sample. If only some extender components are present, additional components can optionally be added so that selected sperm sample includes a complete extender or an extender fraction before the isolation step. In exemplary embodiments, bovine sperm are flow-sorted so as to produce a selected sperm sample including the A fraction of an extender (see Examples 2, 3, and 4). If desired, the B fraction can
20 then be added to the selected sperm sample before the isolation step (see Example 5). The pre-isolation step extender (or extender fraction) is termed "the initial extender" to distinguish it from the "final extender" employed for the extension of isolated sperm before freezing. If the selected sperm sample was selected using FACS, the initial extender can be matched to the sheath fluid employed for sorting. Exemplary matched sheath fluids and extenders are described
25 in detail in Example 4.

An extender suitable for use in the selected sperm sample includes a physiologically acceptable carrier. The physiologically acceptable carrier is typically aqueous, and, in preferred embodiments, includes deionized water. Suitable extenders commonly comprise one or more of the following additional components: a cryoprotectant, a component that maintains osmolality
30 and buffers pH, an organic substance that prevents cold shock and preserves fertility of sperm, a detergent that acts synergistically with certain organic substances to enhance preservation of

sperm, an energy source that can be readily utilized by sperm, an antioxidant, which protects sperm from cold shock, a substance that facilitates sperm capacitation, and one or more antibiotics.

Although cryoprotectants useful in the invention are not limited to those acting by a particular mechanism, most conventional cryoprotectants act, at least in part, by reducing intracellular dehydration. Specifically, freezing is accompanied by an increase in solute concentration in the medium surrounding sperm. This increase in solute concentration draws water out of the cells, which increases intracellular electrolyte concentration. Exemplary cryoprotectants include glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and the like. The cryoprotectant suitable for use in a given extender can vary, depending on the species from which sperm are derived. For example, glycerol is suitable for use in cryopreservation of human and bovine sperm, but is generally not used for cryopreservation of porcine or rabbit sperm. Such preferences are well known for many commercially valuable sperm and can readily be determined empirically for other types of sperm.

The extender useful in the invention optionally includes one or more components that help maintain osmolality and provide buffering capacity. In preferred embodiments of the invention, the osmolality of the extender approximates that of physiological fluids. More preferably, the osmolality of the extender is in the range of about 280 mOsm to about 320 mOsm. The pH is also preferably within a physiologically acceptable range, more preferably in the range of about 6.5 to about 7.5.

Substances helpful in maintaining osmolality and pH within these ranges are well known in the art and can be added to the extender as a solid or already in solution. A buffer containing a salt, a carbohydrate, or a combination thereof can be employed for this purpose. Specific examples include sodium citrate, Tris[hydroxymethyl]aminomethane, and TES (N-Tris [Hydroxymethyl]methyl-2-aminoethanesulfonic acid), and monosodium glutamate buffers; milk; HEPES-buffered medium; and any combination thereof. The component employed to help maintain osmolality and provide buffering capacity in a particular application can vary depending on the other components of the extender and, in some cases, on the species from which the sperm are derived. The selection of such a component for use in the present invention is, however, within the level of skill in the art.

One or more organic substances that protect sperm against cold shock and help preserve fertilizing capacity can also be included in the extender. Such substances are well known and are sometimes described as “nonpenetrating cryoprotectants.” One skilled in the art can readily determine an organic substance suitable for a particular application of the cryopreservation method described herein. For example, organic substances containing protective constituents (e.g., lipoproteins, phospholipids, lecithin) that are believed to reduce the impact of cold shock and the dilution effect can be included in the extender. Suitable organic substances include disaccharides, trisaccharides, and any combination thereof. Exemplary organic substances include egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, cholesterol, and any combination thereof.

The extender can also include a detergent. Alkyl ionic detergents, such as sodium dodecyl sulfate (SDS), have been reported to act synergistically with egg yolk to enhance protection against cold shock. Other detergents useful in the cryopreservation of cells can also be employed in the extender, and the selection of a particular detergent for a specific application is within the level of skill in the art in light of the guidance provided herein. See, e.g., Example 5.

Preferably, the extender includes an energy source that is readily utilized by sperm. In the absence of an energy source, sperm may oxidize intracellular phospholipids and other cellular components. Thus, the inclusion of an energy source in the extender protects intracellular reserves and cellular components. As is well known in the art, sugars, particularly monosaccharides, provide a convenient energy source, although any conventional energy source can be employed in the extender. Exemplary monosaccharides useful in the extender include glucose, fructose, and/or mannose.

One or more antioxidants can optionally be included in the extender to provide additional protection against cold shock. Exemplary antioxidants include butylated hydroxytoluene (BHT), its derivatives, and the like. However, other antioxidants useful in the cryopreservation of cells can also be employed in the extender, and the selection of a particular antioxidant for a specific application is within the level of skill in the art in light of the guidance provided herein.

The extender can also contain a substance that facilitates sperm capacitation. A variety of capacitation facilitators are known in the art and any can be employed in the extender. Examples

include enzymes such as alpha amylase, beta amylase, beta glucuronidase, which can be used in combination, if desired.

Finally, the extender preferably includes an antibiotic, since substantial bacterial growth can threaten sperm viability and increase the risk of infection of the host in artificial insemination or in vitro fertilization procedures. Any of a variety of antibiotics useful in the cryopreservation of cells can also be employed in the extender. The selection of a suitable antibiotic depends on the species from which the sperm was obtained, the procedures involved in obtaining and handling the sperm sample, and the specific microorganism(s) to be targeted. Exemplary antibiotics include tylosin, gentamicin, lincomycin, spectinomycin, linco-spectin (a combination of lincomycin and spectinomycin), penicillin, streptomycin, and ticarcillin, which can be used alone or in combination. However, one skilled in the art can readily determine other antibiotics suitable for use in the extender.

Exemplary extenders are discussed in greater detail below and in the examples.

The sperm concentration is typically lower in the selected sperm sample than in the source sample, and, as indicated above, when FACS is employed, the dilution is significant. A typical sort based on sex-type can produce a sample containing sperm at 6×10^5 cells/ml catch buffer. As such a low concentration is not optimal for storage (at least for most species tested), the cryopreservation method of the invention generally concentrates the selected sperm sample.

Cooling the Selected Sperm Sample

The second step in the cryopreservation method entails cooling the selected sperm sample, typically, by reducing the temperature at a controlled rate. Cooling too rapidly can cause cold shock, which can result in a loss of membrane integrity and cell function. The severity of the effects of cold shock vary from species to species and depend on factors such as the rate of cooling and the temperature range. Under suitable controlled cooling conditions, the sperm are able to adapt to thermal effects. Example 2, among others, describes conditions for cooling bovine sperm, and determining suitable conditions for cooling sperm of other species is within the level of skill in the art.

In a preferred embodiment of the invention, the selected sperm sample is cooled typically from about 22°Celsius, to about 5°Celsius, and cooling is generally carried out over a period of about 60 minutes to about 24 hours, preferably over a period of about 90 minutes to about 240

minutes, and most preferably over a period of about 90 minutes to about 120 minutes. Cooling can be accomplished by any convenient method, including simply placing the selected sperm sample in a 5°Celsius environment.

5 *Isolation of Sperm Cells from the Selected Sperm Sample*

After initial extension of the selected sperm sample, sperm are isolated from the sample using any sufficiently gentle isolation method that provides at least about 50% recovery of sperm, more preferably about 75% to about 90% recovery of sperm, and most preferably about 80% to about 90% recovery of sperm. During the isolation step, the cooled sperm should
10 generally be kept cold, i.e., between about 1 and about 8°Celsius, and preferably close to 4 or 5°Celsius.

Any of a variety of methods suitable for recovering cells from a suspension can be used to isolate the sperm, including for example, filtration, sedimentation, and centrifugation. In an exemplary, preferred embodiment, the selected sperm sample is aliquoted into 50 ml tubes at
15 volumes not exceeding about 27 ml, and preferably between about 20 to about 27 ml. Centrifugation is carried out at about 4°Celsius, at about 850 x g, for about 20 minutes. Preferably, the centrifugation step provides at least about 50% to about 90% recovery of sperm, more preferably about 60% to about 90% recovery of sperm, and most preferably about 70% to about 90% recovery of sperm. After isolation, the supernatant is removed and the pellet is
20 suspended by vortexing gently or repeated aspiration at 4°Celsius. The sperm concentration is then typically determined (e.g., using a hemacytometer).

Final Extension of Isolated Sperm Cells

After isolation, the sperm are pooled, if desired, and extended with final extender to an
25 appropriate concentration for freezing. The concentration of sperm after the final extension and prior to freezing is preferably in the range of about 1×10^6 /ml to about 300×10^6 /ml, more preferably about 10×10^6 /ml to about 50×10^6 /ml, and most preferably about 10×10^6 /ml to about 20×10^6 /ml.

The description of the initial extender above also applies to the final extender, which can
30 be the same as or different from the initial extender. In particular embodiments, the composition

of the sperm sample extended with the final extender is substantially similar to (if not the same as) the composition of the sperm sample after addition of the initial extender.

In a preferred embodiment of the invention, an egg yolk-Tris extender is used. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); citric acid
5 and Tris[hydroxymethyl]aminomethane (buffer); egg yolk (organic substance); fructose (energy source); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

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Components of Egg Yolk-Tris Extender

	Glycerol:	4-8% vol/vol
	Citric Acid:	55-75 mM
	Tris [hydroxymethyl]aminomethane:	190-210 mM
5	Egg yolk:	5-25% vol/vol
	Fructose:	45-65 mM
	Tylosin:	25-100 µg/ml
	Gentamicin:	200-300 µg/ml
	Linco-spectin:	100-400 µg/ml*

* 100-400 µg/ml lincomycin and 100-400 µg/ml spectinomycin

In a variation of this embodiment particularly suitable for freezing bovine sperm, the concentrations of these components after addition of the final extender to the isolated sperm are about 6% (vol/vol) glycerol, about 65 mM citric acid, about 200 mM

Tris[hydroxymethyl]aminomethane, about 20% (vol/vol) egg yolk, about 56 mM fructose, about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 150/300 µg/ml linco-spectin (i.e., 150 µg/ml lincomycin and 300 µg/ml spectinomycin), in deionized water.

In an alternative embodiment, an egg yolk-citrate extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); sodium citrate (buffer); egg yolk (organic substance); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

Components of Egg Yolk-Citrate Extender

Glycerol:	4-8% vol/vol
Sodium Citrate :	60-80 mM
Egg yolk:	5-25% vol/vol
Tylosin:	25-100 µg/ml
Gentamicin:	200-300 µg/ml
Linco-spectin:	100-400 µg/mL *
* 100-400 µg/ml lincomycin and 100-400 µg/ml spectinomycin	

Exemplary, preferred concentrations for freezing bovine sperm are about 7% (vol/vol) glycerol, about 72 mM sodium citrate, about 20% (vol/vol) egg yolk, about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 250/300 µg/ml linco-spectin.

In another alternative embodiment, an egg yolk-TES-Tris ("EY TEST") extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); egg yolk and heated milk, e.g., homogenized milk containing 1.25% fructose with 10% glycerol (organic substances); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

Components of Egg Yolk TES-Tris Extender

	Glycerol:	3-7% vol/vol
	Tris [hydroxymethy-methyl]-2-aminoethanesulfonic acid:	140-170 mM
5	Tris [hydroxymethyl]aminomethane:	60-80 mM
	Egg yolk:	5-25% vol/vol
	Fructose:	5-12 mM
	Tylosin:	50-150 µg/ml
	Gentamicin:	400-600 µg/ml
10	Linco-spectin:	200-700 µg/mL*

* 200-700 µg/ml lincomycin and 200-700 µg/ml spectinomycin

Exemplary, preferred concentrations for freezing bovine sperm are about 5% (vol/vol) glycerol, about 158mM Tris[hydroxymethy-methyl]-2-aminoethanesulfonic acid, about 72 mM
15 Tris[hydroxymethyl]aminomethane, about 20% (vol/vol) egg yolk, about 8mM fructose, about 100 µg/ml tylosin, about 500 µg/ml gentamicin, and about 300/600 µg/ml linco-spectin.

In yet another alternative embodiment of the invention, a Milk extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); heated homogenized milk (organic substance); fructose (energy source); and tylosin, gentamicin,
20 and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

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Components of Milk Extender

	Homogenized Milk	90% (vol/vol)
	Glycerol:	3-7% (vol/vol)
5	Fructose:	1.25 % (wt/vol)
	Tylosin:	50 µg/ml
	Gentamicin:	250 µg/ml
	Linco-spectin:	250/300 µg/ml

*

10 * 250-300 µg/ml lincomycin and 250-300 µg/ml spectinomycin

Exemplary preferred concentrations for freezing bovine sperm are about 90% milk, about 10% (vol/vol) glycerol, about 1.25% fructose (wt/vol?), about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 250/300 µg/ml linco-spectin.

15 Other extenders standardly used to freeze sperm can also be employed as the final extender in freezing selected sperm. A variety of extenders optimized for use in freezing sperm from various species have been described, and many are commercially available. Freezing extenders for equine sperm typically consist of milk, egg yolk, various sugars, electrolytes and a cryoprotectant. Exemplary freezing extenders are described by Squires, E.L., et al., Cooled and
20 Frozen Stallion Semen Animal Reprod. and Biotechnology Laboratory, Bulletin No. 69, Chapter 8, "Seminal Extenders" pp. 49-51 (July, 1999).

Equilibration and Freezing of Sperm

25 Extension of the sperm sample produces a suspension of sperm, which is then transferred into containers for freezing. If the sperm are intended for use in fertilization, the cells are conveniently aliquoted into individual doses sufficient to achieve fertilization. The required dose can vary substantially from one species to the next and is either well-known (e.g., for cattle and horses) or can readily be determined. In the case of sex-selected bovine sperm, convenient doses range from about 1.0×10^6 sperm to about 3.0×10^6 sperm.

30 Any suitable container can be employed for freezing, including, for example, an ampule, a vial, and a straw. Sperm intended for AI are typically frozen in straws (e.g., 0.25 ml or 0.50 ml

straws) designed for use with an insemination gun. Preferably, a bolus of extender is drawn into the straw, followed, in sequence, by air, sperm, air, and extender, so that the sperm are flanked on either side by an air space, which separates the sperm from a bolus of extender at either end of the straw.

5 Prior to freezing, the sperm are generally allowed to equilibrate at about 5°C. Preferably, the sperm are allowed to equilibrate for a period in the range of about 1 hour to about 18 hours, more preferably between about 3 hours and about 18 hours, and most preferably between about 3 hours and about 6 hours (see Example 2).

Following equilibration, any standard freezing method can be employed, provided the
10 freezing rate is not too rapid (i.e., not in excess of about 0.5°C/minute). Preferably, the freezing rate is about 0.5°C/minute. In an exemplary, preferred embodiment, the sperm are placed in static liquid nitrogen vapor, and freezing is carried out in three distinct stages over a period of about 10 minutes. In the first stage of freezing, the sperm are cooled from about 5°C to about -15°C at a rate of about 40°C/minute to about 65°C/minute. In the second stage of freezing, the
15 sperm are cooled from about -15°C to about -60°C at a rate of about 25°C/minute to about 35°C/minute. In the third stage, the sperm are plunged into liquid nitrogen at about -100°C.

Selected Sperm Samples

In addition to a freezing method, the invention provides a frozen sperm sample including
20 sperm selected from a source sample for a particular characteristic. The sperm can be from any species, including any of those discussed above with reference to the freezing method. The invention encompasses frozen sperm selected for any characteristic by any suitable method, such as those described above. Preferred embodiments include frozen sex-selected human, bovine, equine, porcine, ovine, elk, or bison sperm. Sex-selection is preferably carried out using flow
25 cytometry as described generally above.

Also within the scope of the invention is a container containing a frozen sperm sample according to the invention. The container can be formed from any material that does not react with the frozen sperm sample and can have any shape or other feature that facilitates use of the sample for the intended application. For samples intended for use in AI, for example, the
30 container is conveniently a straw (e.g., 0.25 ml or 0.5 ml straw) designed for use with an insemination gun. The container is sealed in any manner suitable for preserving the sample at

the intended storage temperature, which is typically below -80°Celsius. 0.25 ml straws can be sealed, for instance, with PVC powder, ultrasonically, or with a cotton-polyvinyl plug and/or a stainless steel ball (BB).

As the frozen sperm sample of the invention is typically thawed before use, the invention also provides a thawed, previously frozen, selected sperm sample and a container including such a thawed sample.

Methods of Using the Selected Sperm Sample

The frozen selected sperm sample of the invention is suitable for use in any method in which sperm are used. The sample can be thawed and used in any conventional fertilization method, such as artificial insemination or in vitro fertilization. Thawing is carried out in the same manner as for frozen, non-selected sperm. Briefly, the straw containing the frozen sperm is submerged in a water bath maintained at a temperature of about 35°C to about 37°C for a period of about 20 to about 30 seconds. After thawing, semen deposition (e.g., insemination) is carried out according to standard procedures, taking care to protect the sperm from environmental fluctuations.

EXAMPLES

Example 1

Effects Of Dilution On Sperm

- 5 Objective: to determine the effect of sperm concentration on sperm motility for non-frozen, non-sorted, but highly diluted sperm.

A. *Effects of Dilution on Non-washed Sperm*

1. Collection of Source Sample. Sperm were collected from bulls on a routine
10 collection schedule using an artificial vagina as described in Schenk J., Proc 17th NAAB, p.48-58 (1998), and Saacke RG, Proc NAAB Tech Conf AI Reprod. 41:22-27 (1972). All ejaculates used contained greater than 50% progressively motile and greater than 75% morphologically normal sperm. Antibiotics were added to the raw ejaculate as described by Shin S., Proc NAAB Tech Conf AI Reprod. 11:33-38 (1986) within 15 minutes of collection, and the concentration of
15 sperm was determined using a spectrophotometer.

2. Methods. Sperm from 4 bulls were diluted to 1.25, 2.5, 5, 10, 15, and 20×10^6 /ml using an egg yolk-citrate extender (EYC) prepared with 20% egg yolk (vol/vol) in 72 mM sodium citrate, 50 µg/ml tylosin, 250 µg/ml gentamicin, and 250/300 µg/ml linco-spectin. Each
20 sample was prepared in duplicate (2 tubes/dilution/bull) and comprised 8 ml total volume per tube. All samples were incubated for 60 minutes at 22°C, after which they were centrifuged using a swinging bucket centrifuge (Eppendorf, Model # 5810R) at $600 \times g$ for 10 minutes to concentrate the sperm. After centrifugation, the supernatant from one set of the duplicate tubes was not removed; the sperm were resuspended in the same medium and at the original
25 concentration by repeated gentle aspiration using a 5-ml serological pipette. (The second set of the duplicate tubes were used in Example 1B.) Sperm samples were then cooled to 5°C at 0.2°C/min over 90 minutes. These sperm were termed “non-washed sperm.” All samples were incubated at 5°C for 24 or 48 h post-collection.

3. Evaluation of Motility. After incubation, the samples were warmed to 37°C using
30 a dry block incubator for 10 minutes prior to determination of motility. For this experiment, a

single, blind estimate of the percentage of progressively motile sperm was determined for each sample. Progressive sperm motility was determined subjectively for each subclass by a single observer (x200, phase-contrast microscopy); another person prepared the microscope slides in a randomized manner so the observer was unaware of treatments.

4. Statistical Analysis. Data were analyzed by analysis of variance (SAS Institute, Cary, North Carolina) with factors bulls and initial dilution concentration. Separate analyses were done for each incubation time. Dilution trends were tested using (log) linear contrasts.

5. Results. Data for non-washed sperm (Table 1) revealed (log) linear relationships (P<0.01) for both incubation times. Percentages of motile sperm increased as sperm concentration increased from 1.25 x 10⁶/ml to 10 x 10⁶/ml, but there was little difference thereafter. The cubic term was significant (P<0.05) for 24-h and marginally significant (P<0.1) for 48-h incubations. There was a bull effect (P<0.01) at both times.

Table 1. Effects of cooling on non-washed sperm motility (%) after cooling to 5°C.

Dilution (10 ⁶ /ml)	Incubation at 5°C	
	24 h ^a	48 h ^b
1.25	18 ^c	0 ^c
2.5	38 ^{c,d}	6 ^{c,d}
5.0	56 ^d	31 ^{d,e}
10.0	61 ^d	42 ^e
15.0	55 ^d	44 ^e
20.0	58 ^d	41 ^e
S.E. ^f	5.6	6.4

^a (log) linear (P<0.01) and cubic effects (P<0.05).

^b (log) linear (P<0.01) and cubic effects (P<0.1).

^{c,d,e} Means within columns without common superscripts differ (P<0.05).

^f $\sqrt{\text{error mean square of ANOVA} - \sqrt{N}}$
(SAS Institute, Cary, NC, USA)

B. Effects of Dilution on Washed Sperm

1. Collection of Source Sample. The second set of the duplicate tubes containing samples prepared in Example 1A were used in this experiment.

2. Methods. The sperm were diluted, incubated and concentrated by centrifugation as in Example 1A. Following centrifugation, 7.1 ml of the supernatant was aspirated from each tube, removing most of the seminal plasma and leaving the sperm in a 900-μl pellet. The sperm were diluted with EYC (see Example 1A) to make 10 × 10⁶/ml or 20 × 10⁶/ml sperm suspensions. The samples were then cooled to 5°C over 90 minutes as in Example 1A.

3. Evaluation of Motility. The samples were warmed and evaluated for progressive motility as in Example 1A.

4. Statistical Analysis. Data were analyzed as in Example 1A. In addition, data in Example 1B were analyzed for incubation concentration at 5°C.

5. Results. Data for washed sperm (Table 2) revealed no significant treatment effects when sperm were evaluated after 24 h. However, after storage for 48 h at 5°C, there were bull, initial dilution, incubation concentration and bull by incubation effects (P<0.05). More sperm remained motile when held at 20 × 10⁶ /ml than at 10 × 10⁶ /ml (31% vs. 20%; P<0.05). Initial dilutions of 1.25, 2.5, and 5 × 10⁶ sperm/ml resulted in lower progressive motility than 10 × 10⁶ sperm/ml (P<0.05), with respective main effect means of 19, 20, 27, and 37% motile sperm.

Table 2. Cumulative effects of washing, dilution, concentration and cooling on progressive sperm motility (%)

Sperm conc (10 ⁶ /ml) during 1 h preincubation at 37°C	Storage at 5°C – Sperm Concentration and Duration			
	24 h		48 h ^a	
	20 × 10 ⁶ /ml	10 × 10 ⁶ /ml	20 × 10 ⁶ /ml	10 × 10 ⁶ /ml ^b
1.25	45	49	24	15
2.5	51	40	29	11
5.0	54	54	32	21
10.0	51	50	40	34
15.0	60		41	
20.0	55		40	

^a Concentration to 20 × 10⁶ sperm/ml was superior (P<0.05) to 10 × 10⁶ sperm/ml after 48 h storage.

Also, initial dilution to 10 x 10⁶ was superior to lower dilutions (P<0.05).

Pooled standard errors ($\sqrt{\text{error mean square of ANOVA}} - \sqrt{N}$ were 4.0 for 24 h, and 2.8 for 48 h incubations.

^b Significant (log) linear trend (P<0.06).

C. Conclusion

High sperm dilution and cooling resulted in a substantial reduction in the percentage of motile sperm, regardless of the presence or removal of seminal plasma. However, this dilution

effect was greatly attenuated by concentrating the diluted sperm to $10 \times 10^6/\text{ml}$ and even more, to $20 \times 10^6/\text{ml}$ before storage at 5°C . Sperm from some bulls tolerated dilution better than sperm from other bulls; however, the bull differences found are typical. Extremely dilute sperm might be compromised during sorting, in part, by removal of protective compounds in seminal plasma.

5

Example 2

Effects of Equilibration Time Before Freezing Sorted Sperm

Objective: to evaluate the effect of equilibration times (3, 6 and 18 h, 5°C) before freezing on flow-sorted sperm.

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The following experiment was replicated in its entirety using the same bulls:

1. Collection of Source Sample. Sperm of 4 bulls were collected and prepared as described in Example 1A.

15

2. Methods.

a) Staining and Preparation for Sort.

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i) Preparation of Stain Stock Solution: a stock solution of 8.89 mM Hoechst 33342 (bis-Benzimide H-33342; #190305, ICN Biomedicals Inc., Aurora, OH) was prepared in deionized water.

25

ii) Sperm Stain Procedure: sperm were diluted in a modified TALP buffer (Table 3) to 400×10^6 sperm/ml. Following dilution, Hoechst 33342 dye was added to the sperm suspensions at a concentration of $224 \mu\text{M}$. After the stain was added to the sperm suspensions, the samples were incubated for 60 minutes at 34°C . Following incubation, sperm were diluted to $100 \times 10^6/\text{ml}$ with TALP containing 2.67% clarified egg yolk and 0.002% food coloring dye (FD&C #40) which quenches the fluorescence of Hoechst 33342 in sperm with compromised cell membranes, allowing them to be gated out during the sorting process. Just prior to flow sorting, samples were filtered at unit gravity through a $40\text{-}\mu\text{m}$ nylon mesh filter to remove any debris and/or clumped sperm.

30

b) Sorting. A two-line argon laser operating at 351 and 364 nm and 150 mW was used to excite the Hoechst 33342 dye. The flow cytometer/cell sorter used was an SX MoFlo® (Cytomation, Inc., Fort Collins, CO, USA) operating at 50 psi. A Tris-based sheath fluid was used, consisting of Tris (hydroxymethyl) aminomethane (Tris; 197.0 mM; #T-1503, Sigma Chemical Co., St. Louis, MO, USA), citric acid monohydrate (55.4 mM; #C-7129, Sigma Chemical Co., St. Louis, MO, USA) and fructose (47.5 mM; #F-0127, Sigma Chemical Co., St. Louis, MO, USA). Baseline antibiotics were also added to the Tris-based sheath fluid consisting of 0.58 g/L of penicillin and 0.05 g/L of streptomycin sulfate.

The sperm were sorted by a process referred to as “bulk sorting” which permits rapid accumulation of large numbers of sperm so that large-scale examples can be done within a reasonable time. The sperm pass through the flow cytometer under the standard operating conditions with the exception that all droplets containing viable sperm were collected into a single tube rather than being sorted into 2 tubes based upon specific characteristics (e.g., sorting by sex-type). Sperm were sorted on the basis of viability; hence, sperm that have compromised plasma membranes were excluded during bulk sorting.

Stained sperm were maintained at $22 \pm 1^\circ\text{C}$ during sorting. Bulk sorted sperm were collected in 50-ml plastic tubes containing 2 ml of 20% egg yolk-Tris extender prepared with 20% egg yolk (vol/vol) in 200 mM Tris, 65 mM citric acid, 56 mM fructose, 50 µg/ml tylosin, 250 µg/ml gentamicin, and 150/300 µg/ml linco-spectin in deionized water. The egg yolk-Tris extender was termed “Tris-A fraction” to denote the lack of glycerol at this point in the procedure. Sperm were collected in tubes to contain 12 ml and approximately 6×10^6 sperm. The sperm were subsequently incubated at 22°C for 1 to 3 h to simulate conditions of a sort based on sex-type.

c) Preparation for Freezing. Following incubation, the sorted sperm were cooled to 5°C over the period of 70 minutes. After cooling, the contents of the two tubes were pooled and transferred to a refrigerated, swinging bucket centrifuge set at 5°C and

centrifuged at $850 \times g$ for 20 minutes. After removing the supernatant, processing continued at 5°C by adding about 150 μl of Tris-A fraction extender to about 150- μl of sperm pellet to bring the sperm concentration to approximately $40 \times 10^6/\text{ml}$. The sperm of individual bulls were pooled and diluted immediately with an equal volume of egg yolk-Tris extender containing 12% (v/v) glycerol ("Tris-B fraction"). The Tris-B fraction was added to the sperm suspension as 2 equal volumes at 15-minute intervals to adjust the final sperm concentration to $20 \times 10^6/\text{ml}$. The final glycerol concentration of the complete egg yolk-Tris extender containing the sperm was 6% (v/v).

d) Equilibration and Freezing. Extended sperm were then packaged into 0.25-ml polyvinylchloride straws to be frozen by routine procedures on racks in static liquid nitrogen vapor. Two straws from each of 4 bulls were frozen after 3, 6 and 18 h of total equilibration time at 5°C .

3. Evaluation of Post-Thaw Motility. Straws were thawed in a 37°C water bath for 30 sec. Blind estimates of progressive motility were made after incubating samples at 37°C for 0, 1 and 2 h post-thawing. Each of two observers estimated progressive sperm motility from each of two straws of semen. These four blind estimates for each experimental unit represent subsampling.

4. Statistical Analysis. Statistically, the subsamples were analyzed as a subplot to the main plot least-squares ANOVAs to analyze effects of any observer and observer x treatment interaction. N refers to the number of experimental units, not subsamples; standard errors were calculated on the basis of means of the 4 subsamples from error mean squares of ANOVAs and the numbers of experimental units; least-squares means are presented.

Treatment effects were evaluated via separate ANOVAs for each incubation time. The model included bulls as a random effect and equilibration time and observer as fixed effects; the subplot consisted of the observer term and related interactions.

5. Results. The 3- or 6-h equilibration times were superior to 18-h (Table 4), based on the percentage of progressively motile sperm, for 0 and 1 h (P<0.01) but not 2 h of post-thaw incubation. Effects of bull were evident at 1 and 2 h incubation times (P<0.05), but not at 0 h. There was no significant (P>0.1) bull by equilibration time interaction nor was there a significant observer effect for any response.

Table 3. Modified TALP Buffer

Ingredient	Concentration
NaCl	95.0 mM
KCl	3.0 mM
NaHPO ₄	0.3 mM
NaHCO ₃	10.0 mM
MgCl ₂ • 6H ₂ O	0.4 mM
Na Pyruvate	2.0 mM
Glucose	5.0 mM
Na lactate	25.0 mM
HEPES ^a	40.0 mM
Bovine serum albumin ^b	3.0 mg/ml
Gentamycin Sulfate	30.0µg/ml

^a #H3375, Sigma Chemical Co., St. Louis, MO, USA

^b #US70195, fraction V; Amersham/Life Science, Cleveland, OH, USA

Table 4. Effect of pre-freeze equilibration time on post-thaw progressive motility (%)

Equilibration at 5°C	Post-thaw incubation at 37°C		
	0 h	1 h	2 h
3 h	41 ^a	36 ^{a,b}	16
6 h	41 ^a	37 ^a	18
18 h	35 ^b	31 ^b	12
S.E. ^c	1.5	0.8	2.0

^{a,b} Within columns, means without common superscripts differ (P<0.05), Tukey's HSD.

^c Pooled standard errors,
 $\sqrt{\text{error mean square of ANOVA}} - \sqrt{N}$

6. Conclusion. The results indicated no differences in post-thaw sperm motility between 3 and 6 h of total equilibration time at 5°C, but there was a significant decline in sperm motility following 18 h of equilibration at 5°C before freezing. The 3- to 6-h range permits pooling 2 consecutive 3-h sorting batches for freezing sperm without decreasing post-thaw motility.

As the bull by equilibration-time interaction was not significant, 3 to 6 h equilibration was adequate, with the caveat that only 4 bulls were used. The optimum equilibration time for a minority of bulls is expected to be >6 h.

Example 3

Effects Of Stain Concentration And Laser Power on Sorted Sperm

Objective: to evaluate the effects of Hoechst 33342 dye concentration in combination with laser intensity on flow-sorted sperm.

1. Collection of Source Sample. Sperm of 6 bulls were collected and prepared as described in Example 1A.

2. Methods.

a) Experimental Design. One ejaculate (2 bulls) and 2 ejaculates on different days (4 bulls) were used in a 2 by 2 design plus control.

b) Staining and Sorting. Staining, preparation for sorting and sorting sperm were achieved as described in Example 2 except that the Hoechst 33342 dye was added to sperm suspensions at a final concentration of 149 μM or 224 μM ; and sperm were bulk-sorted with the laser operating at 100 mW or 150 mW of incident power. Bulk-sorted sperm were collected into 50-ml plastic tubes as described in Example 2. Four tubes containing approximately 15×10^6 total sperm/tube were collected over 1 h for each bull. The sorted sperm were incubated for 1 h at 22°C to simulate a longer sorting time.

c) Preparation for Freezing. Following incubation, the sperm were cooled as in Example 2. The sperm were then concentrated by centrifugation at 5°C at $850 \times g$ for 20 minutes. After removing the supernatant, 150 μl of Tris-A fraction extender was added to each 150- μl sperm pellet at 5°C. All of the sperm pellets were suspended by gentle repeated aspiration and the sperm of individual bulls were pooled. Tris B-fraction extender was added stepwise as described in Example 2. A non-stained, non-sorted control for each bull was prepared at 20×10^6 sperm/ml in Tris extender containing 6% glycerol and cooled to 5°C while the bulk-sorted sperm were being prepared.

d) Equilibration and Freezing. The control and sorted sperm were packaged into 0.25-ml polyvinylchloride straws as described in Example 2, equilibrated at 5°C for 3 h and then frozen conventionally.

3. Evaluation of Post-Thaw Motility. Straws were thawed and evaluated as described in Example 2.

4. Statistical Analysis. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via ANOVA. The model included dye concentration, laser intensity and bulls in the main plot, and observer and related interactions in the subplot. Bulls were considered a random effect and the other factors as fixed.

5. Results. Bull effects were significant for percentages of progressively motile sperm immediately after thawing ($P<0.1$) and after 1 h and 2 h of incubation at 37°C ($P<0.05$). There was no effect of dye concentration or bull by dye concentration on sperm motility at any incubation time. With bulls considered as a random effect, 150 mW of laser power resulted in lower post-thaw motility of sperm than 100 mW at 0 h of incubation ($P<0.1$), but not at other incubation times (Table 5). If bulls are considered as fixed effects, 150 mW of power resulted in lower sperm motility than 100 mW ($P<0.05$) at all 3 incubation times. There was an effect of bull by laser power ($P<0.05$) on sperm motility at 1 h, but not at 0-h or 2-h incubation times. Also, the higher laser power resulted in lower sperm motility than the control ($P<0.05$) at 0- and 1-h incubation times (Table 5). There was a significant observer effect at 1-h, but not at 0-h or 2-h, incubation times. There was no observer by treatment interaction ($P>0.1$).

Table 5. Effects of laser intensity and dye concentration on post-thaw motility (%).

Main effect means	Incubation at 37°C		
	0 h	1 h	2 h
Control	49	44	33
<u>Dye Concentration</u>			
149 µM	41	39	30
224 µM	42	39	30
<u>Laser Intensity</u>			
100 mW	46	42	33
150 mW	38 ^a	35 ^b	27
S.E. ^c	2.2	1.2	1.3

^a Significant main effect (P<0.1) and differs from control (P<0.05).

^b Differs from control (P<0.05).

^c Pooled standard errors, $\sqrt{\text{error mean square of ANOVA} / N}$

6. Conclusion. Percentages of progressively motile sperm post-thaw were diminished by the staining and sorting process. Higher laser intensity was more damaging than the lower laser intensity. There was no effect of dye concentration on post-thaw sperm motility. Thus, excitation of the sperm-bound Hoechst 33342 dye at lower laser intensities is less damaging and that staining sperm at the higher dye concentration had no detrimental effect on post-thaw motility. The damage observed was presumably to the sperm-motility apparatus.

Example 4

Evaluation of Pre-Sort Staining Procedures and Selection of Extenders for the Cryopreservation Of Sperm

Objective: (1) to evaluate three pre-sort treatments for sperm; and, (2) and to evaluate sheath
5 fluid and extender combinations for the cryopreservation of flow-sorted sperm.

The following experiment was replicated in its entirety:

1. Collection of Source Sample. Sperm from 4 bulls were collected and prepared as
described in Example 1A.

10 2. Methods.

a) Experimental Design. A 3 (pre-sort treatments) by 3 (extenders) by 2
(sheath fluids) by 4 (bulls) by 2 (observers) factorial experiment was designed to
determine the best procedure to hold sperm prior to sorting, and to evaluate three
15 extenders for cryopreserving the sorted sperm. .

b) Sample Preparation and Staining. Freshly collected sperm from each of 4
bulls were treated as follows:

(1) diluted to 400×10^6 /ml in modified TALP (see Example 2, Table 3)
20 and stained for 1 h at 34°C before bulk-sorting (“Dilute – 0 h”);

(2) incubated neat at 22°C for 3 h before dilution, staining and sorting
25 (“Neat – 3 h”); or,

(3) diluted and stained as “Dilute-0 h” and then incubated at 22°C for 3 h
before bulk-sorting (“Diluted – 3 h”).

c) Extenders. The following freezing extenders were compared: EYC (see
Example 1) containing 7% glycerol, egg yolk-Tris (see Example 2) containing 6%
glycerol, and egg yolk-TES-Tris (TEST) containing 5% glycerol. EYC “A
30 Fraction” refers to the EYC extender containing no glycerol, and EYC “B
Fraction” refers to EYC extender containing twice the final, desired glycerol

concentration (i.e., 14%). Thus, when EYC A and B fractions are combined in equal volume, the final EYC extender contains 7% glycerol. Tris A and B fractions are similarly named, and described in Example 2. TEST extender is prepared as a complete extender containing 5% glycerol; hence, there were no “A” and “B” fractions for TEST.

d) Sheath Fluid. Sheath fluid was either 98.6 mM sodium citrate dihydrate (#S279-3, Fisher Scientific, Fair Lawn, NJ) or Tris as described in Example 2. Both types of sheath fluid were adjusted to pH 6.8; osmolality was about 270 to 280 mOsm/kg. Tris sheath fluid was used to collect sperm that were later extended in egg yolk-Tris and TEST freezing extenders. Sheath fluid containing 98.6 mM sodium citrate dihydrate was used to collect sperm to later be extended in EYC freezing extender.

e) Sorting. Approximately 58×10^6 sperm for each combination of pre-sort treatment, sheath fluid and extender were bulk-sorted as described in Example 2 using 150 mW of incident laser power. For each sort, sperm were collected over approximately 1 h. After sorting, the samples were incubated at 22°C for 2 h to simulate a 3 h sort.

f) Preparation for Freezing. Following incubation, the sperm were cooled as described in Example 2. After cooling, the samples were centrifuged at 5°C at $850 \times g$ for 20 min. Each sample comprised about 28 ml total volume and was contained in a 50-ml plastic tube

After the supernatant was removed, the sperm were returned to a 5°C cold room for extension. Samples were extended to 40×10^6 /ml by depositing 131 μ l of the sperm suspension into 69 μ l of A-fraction EYC, A-fraction egg yolk-Tris, or TEST extender. Immediately, suspensions were adjusted to 20×10^6 sperm/ml with the addition of the matched glycerol containing extender (i.e., B-fraction EYC, B-fraction Tris) or TEST. B-fraction extenders were added to their respective samples stepwise (2X) at 15-min intervals as described in Example 2.

The TEST was added to sperm stepwise in the same manner as B-fraction EYC and Tris extenders.

g) Equilibration and Freezing. Sperm were packaged into 0.25-ml polyvinylchloride straws, equilibrated for 3 h at 5°C and then frozen in static liquid nitrogen vapor.

3. Evaluation of Post-Thaw Motility. Thawing and post-thaw evaluations of sperm were done as described for Example 2.

4. Statistical Analysis. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via separate analyses of variance for each post-thaw incubation time. The main plot included pre-sort treatment, extenders, and bulls; the subplot consisted of observers and associated interactions. Bulls were considered a random effect, and the other factors, fixed. The entire experiment was replicated twice. Tukey's HSD test was used to separate means.

5. Results. Post-thaw progressive motility of bulk-sorted sperm was affected ($P<0.05$) by extender and bulls at each post-thaw incubation time and by pre-sort procedure at 0 h of incubation (Table 6). There were no differences due to sheath fluids ($P>0.05$). At 0-h post-thaw incubation, use of the neat-3h treatment resulted in more motile sperm after freezing and thawing than the other 2 pre-sort staining treatments ($P<0.05$; Table 6). However, pre-sort procedures were not statistically significant after post-thaw incubation of sperm for 1 or 2 h with bulls considered as a random effect. Importantly, at these 2 incubation times, there were significant pre-sort treatment by bull interactions ($P<0.05$). Furthermore, pre-sort treatment would have been a significant effect at all post-thaw incubation times had bulls been considered as fixed effects.

Immediately after thawing (0 h), TEST was the best extender, but after 1 or 2 h of incubation of 37°C, Tris was the best extender. Importantly, there was no pre-sort treatment by extender interaction for any response. There were observer effects ($P<0.01$) at all incubation

times, but no observer by treatment interactions. There was a bull by extender interaction (P<0.05) at all 3 incubation times.

Table 6. Main effects of pre-sort treatment and freezing extenders on post-thaw progressive motility (%)

Pre-sort procedure	Extender	Incubation at 37°C		
		0 h	1 h	2 h
Dilute – 0 h	Mean	39 ^a	32	22
Neat – 3 h	Mean	43 ^b	36	25
Dilute – 3 h	Mean	38 ^a	31	19
Mean	EYC	36 ^a	29 ^a	17 ^a
Mean	Tris	40 ^b	39 ^b	29 ^b
Mean	TEST	44 ^c	33 ^c	20 ^a
S.E. ^d		0.8	0.8	0.7

^{a,b,c} Means within columns, within main effects, without common superscripts differ (P<0.05).

^d Pooled standard errors = $\sqrt{\text{error mean square of ANOVA} / N}$

6. Conclusion. This study showed that holding sperm neat for 3 h before dilution, staining and sorting was better than immediate dilution and staining 0 h or 3 h later. Thus, by 3 h into the sort, it is best to continue with a new aliquot of the original ejaculate that was held neat 3 h and then stained, rather than continuing with the original sample of sperm stained and held at 400 x 10⁶ sperm/ml.

Even though TEST extender provided higher post-thaw motility at 0 h, Tris was the superior extender when sperm were stressed by incubation at 37°C. Either sheath fluid worked equally well for each extender. Based on these results, we have incorporated the use of Tris sheath fluid in combination with Tris freezing extender into our standard operating procedure.

Example 5

Effects Of Extender Additives on Sorted Sperm

Objective: to evaluate the effect of adding sodium dodecyl sulfate ("SDS") to the freezing extender on flow-sorted sperm.

5

A. *Evaluation of Effect of Concentration of SDS in Freezing Extender*

1. Collection of Source Sample. Sperm of 6 bulls were collected and prepared as described in Example 1A.

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2. Methods. Sperm from each of 6 bulls were extended to 20×10^6 /ml in 20% whole egg Tris ("WET") extender containing 0, 0.03, 0.06, 0.09, or 0.12 percent SDS, packaged into straws and frozen. WET extender was prepared using 3.028 g of Tris[hydroxymethyl] aminomethane, 1.78 g of citric acid monohydrate, and 1.25 g of fructose per 100 ml of double distilled water, to which 20% whole egg (vol/vol) was added. The WET extender was prepared at a pH of about 7.0 and contained a final glycerol concentration of about 6% (vol/vol). The WET extender also contained 1000 IU of penicillin "G" sodium and 100 µg of streptomycin sulfate/ml.

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3. Results. The respective means (n = 1 sample from each of 6 bulls) were 51, 51, 50, 51, and 48% progressive motile sperm approximately 10 minutes post-thaw. Based on these results, 0.06 percent SDS was used in Example 5B.

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B. *Evaluation of the Effects of 0.06 Percent SDS in Various Freezing Extenders on Post-Thaw Motility of Flow-Sorted Sperm*

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1. Collection of Source Sample. Sperm of 8 bulls were collected and prepared as described in Example 1A.

2. Methods. Post-thaw motility was studied for sperm frozen in egg yolk-Tris (see Example 2) and WET extenders (see Example 5A) with and without 0.06% SDS. Final glycerol content for both extenders was 6%.

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a) Staining, Preparation for Sort, Sorting. Stained sperm samples were prepared from an ejaculate from each of 8 bulls as described in Example 2. Stained sperm were bulked-sorted using Tris sheath fluid as described in Example 2 except that the sort was achieved using 135 mW of incident laser power. Sorted sperm were collected in a 50-ml plastic tube containing 2 ml of A-fraction freezing buffer for each extender; 15×10^6 total sorted sperm (25 ml) for each treatment were collected and incubated for 1 h at 22°C to simulate longer sorting.

b) Preparation for Freezing. Diluted sperm were then cooled to 5°C over 90 minutes. An equal volume of appropriate B-fraction extender was added stepwise (2x) at 15-minute intervals to each 50-ml plastic tube containing sorted sperm. Aliquots of 25 ml/extender treatment were concentrated by centrifugation for 20 minutes at 850 x g in a refrigerated centrifuge. The supernatant was removed leaving a 600 µl sperm pellet, which was suspended by gentle vortexing for 15 seconds. No additional extender was added to the sperm pellet since the suspension containing the pellet already contained glycerol. The concentration of the sperm suspension was approximately 20×10^6 /ml. A non-stained, non-sorted control for each bull was prepared at 20×10^6 sperm/ml in egg-yolk-Tris extender containing 6% glycerol. The control was placed in a 5°C cold room while bulk-sorting occurred.

c) Equilibration and Freezing. All control and bulk-sorted sperm were packaged and frozen at the same time. Sperm were packaged into 0.25-ml polyvinylchloride straws, equilibrated for about 3 h to about 6 h at 5°C and then frozen in static liquid nitrogen vapor.

3. Evaluation of Post-Thaw Motility. Thawing and post-thaw evaluations of sperm were done as described for Example 2 with the exception that progressive motility was evaluated 0.5 and 2.0 h after incubation.

4. Statistical Analysis. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via separate analyses of variance for each incubation time; the model included bull and extender in the main plot and observer and

related interactions in the subplot. Differences in means were determined by the least significant difference test.

5. Results. Extender affected ($P<0.05$) progressive motility of sperm after 0.5 or 2 h post-thaw incubation (Table 7). At 0.5 h, WET plus SDS resulted in lower motility than Tris with SDS. At 2 h, all treatments with bulk-sorted sperm were worse than the non-sorted control sperm. There were significant bull and observer effects ($P<0.01$) at both incubation times, but no observer by treatment interactions.

Table 7. Effect of extender on post-thaw progressive motility (%)

Extender	Incubation at 37°C	
	0.5 h	2 h
Tris (non-sort)	42 ^a	41 ^a
Tris w/o SDS	40 ^{a,b}	35 ^b
Tris w/SDS	42 ^a	37 ^b
WET w/o SDS	40 ^{a,b}	35 ^b
WET w/SDS	38 ^b	35 ^b
S.E. ^c	1.0	1.2

^{a,b} Means within columns without common superscripts differ ($P<0.05$).

^c $\sqrt{\text{error mean square of ANOVA} - \sqrt{N}}$

6. Conclusion. The inclusion of SDS in Tris or WET extenders did not benefit sperm quality as determined by visual estimates of motility after thawing. Also, results using WET and Tris extenders were similar; hence, WET appeared as efficacious as Tris for cryopreserving sorted bovine sperm.

Example 6

Quality of Sperm Sexed by Flow Sorting for Field Trials

Objective: to evaluate post-thaw quality of sorted sperm based on acrosomal integrity.

5 1. Collection of Source Sample. Sperm of 3 bulls were collected and prepared as described in Example 1A.

 2. Methods. Sorted and non-sorted control sperm from the same ejaculate were stained, processed, and sorted as described in Example 2 except the sperm were sorted for sex-
10 type at a 90% purity level. Sorted sperm were collected to a volume of approximately 20 ml and were cooled to 5°C for 90 minutes (0.2°C/min). After cooling, an equal volume of egg yolk-Tris B extender (see Example 2) was added to the sorted sperm in 2 equal volumes at 15-minute intervals. Centrifugation and aspiration of the supernatant were achieved as described in Example 5. After centrifugation and aspiration, egg yolk-Tris extender containing 6% glycerol
15 (v/v) was added to the sperm pellet to bring the concentration of sperm to about 20×10^6 /ml. Freezing and thawing were done as described in Example 2 except that equilibration time was about 3 h.

 3. Evaluation of Post-Thaw Motility. Visual estimates of the percentage of
20 progressively motile sperm at 37°C were made approximately 10 minutes after thawing. The acrosomal integrity of sperm was assessed using differential interference-contrast microscopy (x1000) after 2 h of incubation at 37°C. Sperm were treated with 40 mM sodium fluoride, a wet was smear made, and 100 sperm per treatment were examined. Acrosomes were classified as: (a) intact acrosome, (b) swollen or damaged acrosome, or (c) missing acrosome (non-intact).

25 4. Statistical Analysis. The data analyzed were from 19 different freeze dates balanced across 3 bulls used in field trials. Treatment effects (sort vs. control) were evaluated via analysis of variance with bulls as a fixed effect.

 5. Results. The percentage of progressively motile sperm post-thaw was
30 significantly higher ($P < 0.05$) for non-sorted sperm (50%) than for sorted sperm (46%; Table 8), despite removal of dead sperm during sorting. However, the percentage of sperm with an intact

acrosome was not different. Sorting increased the percentage of sperm missing an acrosome, but also reduced the percentage of sperm with a damaged acrosome, relative to control sperm (P<0.05). There were significant differences among bulls for percent of intact acrosomes (P<0.05), percent of non-intact acrosomes (P<0.01), and post-thaw progressive motility (P<0.01). There was a bull by sorting effect for post-thaw motility (p<0.01) but not for the other responses. From bulls A and B, differences in post-thaw motility between sorted and unsorted sperm were near zero; for bull C, sorted sperm were 10 percentage points (19%) lower in motility than control sperm.

Table 8. Effect of sorting on post-thaw motility (%) and acrosomal status (%).

	Acrosomal status			Post-thaw motility
	Intact	Damaged	Non-intact	
Control	64 ^a	20 ^a	15 ^a	50 ^a
Sorted	65 ^a	14 ^b	21 ^b	46 ^b

^{a,b} Column means with different superscripts differ (P<0.05).

6. Conclusion. Visual estimates of progressive motility for sorted, frozen sperm on average were slightly lower (4 percentage points; 8%) than for control sperm, although this difference was larger for one bull. These evaluations were made approximately 10 minutes after thawing. The small average difference is consistent with that for non-intact acrosomes after 2 h of incubation. Sperm with a damaged or missing acrosome are likely to be immotile. The increased percentage of sperm with a non-intact acrosome, for sorted samples, indicates damage associated with sorting or with cryopreservation before or after actual sorting. Presumably, sorting converted damaged acrosomes to missing acrosomes. Based on standard procedures for evaluation of sperm quality, there is no basis for assuming that fertilizing potential of these flow-sorted sperm should be severely compromised for most bulls.

Example 7

Sex-Selection and Cryopreservation of Bull Sperm Using 20% Egg Yolk-Tris Extender

Objective: to provide a protocol for the cryopreservation of flow-sorted bull sperm.

5 1. Collection and Ejaculate Assessment. Collect and prepare ejaculates as described in Example 1A. Select ejaculates from those bulls with >75% morphologically normal sperm. Visually estimate the percentage of progressively motile sperm (ejaculates that have progressive motility >60% are best for sorting). Add antibiotics to raw semen as follows: tylosin at a final concentration 100 µg/ml, gentamicin at a final concentration of 500 µg/ml, and linco-spectin at a
10 final concentration of 300/600 µg/ml.

 2. Staining and Preparation for Sort. Following the addition of the antibiotics to the raw semen sample, allow 15-20 minutes before staining. Stain samples as described in
15 Example 2.

 3. Sorting. Sort for both X- and Y- type sperm, setting the sorting gates for 90% purity. Sort sperm into 50-ml Falcon tubes containing 2 ml 20% egg yolk-Tris A-fraction extender (see Example 2) until each tube contains a maximum of 20 ml total volume (or a maximum of 2 h per sort) and final sorted sperm concentration is 6×10^5 /ml. Note that
20 additional 20% egg yolk-Tris-A fraction catch buffer must be added after the sort and prior to cooling so that the final percentage of egg yolk is at least 3%.

 4. Preparation for Freezing. Following the sort, cool the sorted samples to 5°C over a period of 90 minutes. After cooling, add 20% egg yolk-Tris B-fraction extender (see Example
25 2) stepwise (2X) at 15 minutes intervals. The final volume of Tris B-fraction extender added to the sperm sample should be equal to the volume of Tris A-fraction extender. The total volume of sperm sample after the Tris B-fraction extender is added should not exceed 27 ml total volume.

 After the Tris B-fraction extender is added to the sperm sample, concentrate the
30 sample by centrifugation for 20 minutes at $850 \times g$. Aspirate the supernatant leaving approximately 150 µl sperm pellet. Resuspend the sperm and pool the sperm for each individual bull.

5. Freezing. Add complete egg yolk-Tris extender (6% glycerol) to achieve a final sperm concentration of $20 \times 10^6/\text{ml}$. Package the extended sperm into 0.25-ml polyvinylchloride straws for freezing as described in Example 2.

Example 8

Evaluation of the Fertility of Flow-sorted, Frozen Bull Sperm in Field Studies

MATERIALS AND METHODS

Semen Collection and Processing

Semen from young bulls of unknown fertility was collected via artificial vagina (see Example 1A). After determining sperm concentration with a spectrophotometer and subjective evaluation of progressive sperm motility, semen was processed and sorted as described in Example 2 except that the sperm were sorted by sex-type at 90% purity using a laser incident power of about 135 to about 150 mW. Processing and freezing was achieved as in Example 2 except that the equilibration time was about 3 h. Cornell Universal Extender (Seidel GE Jr., Theriogenology 1997; 48:1255-1264) was used for liquid semen in field trials 1, 2, and 3. For frozen semen in field trials 2 and 3, the extender used was 2.9% Na citrate + 20% egg yolk with a final glycerol concentration of 7% (see Example 1). For field trials 4 through 11, sperm were frozen in a Tris-based extender composed of 200 mM Tris, 65 mM citric acid, 56 mM fructose, 20% egg yolk, and a final glycerol concentration of 6% (see Example 2). The sheath fluid used in the flow cytometer was 2.9% Na citrate (see Example 4) for trials 1, 2, and 3, and a Tris buffer for the remaining trials (see Example 2).

Sperm were packaged in 0.25-ml French straws in columns as small as 50 μl in the center of the straw. To minimize dilution effects, low volumes were used so there were at least 10^7 sperm/ml. In most trials, a column of extender without sperm was aspirated into the straw first to wet the cotton plug, followed by a small column of air, and then the sexed sperm. When sperm were frozen, one straw from each batch was thawed in 35°C water for 30 sec for quality control, and batches with less than 25% progressive motility post thaw were discarded. A sample of sexed sperm from each batch was sonicated and analyzed by flow cytometry to determine the accuracy of sexing.

Heifer Management and Artificial Insemination

The heifers used were in 6 widely scattered production units with different management practices. Seasonal and breed differences contributed further to the heterogeneity of the experiments (Table 9). Insofar as possible, treatments and controls were alternated systematically within bulls within inseminators as heifers entered the insemination facilities.

Estrus was synchronized in one of 4 ways (Table 9): (1) 500 mg of melengesterol acetate (MGA) fed daily in 2.3 kg of grain for 14 days followed by an i.m. injection of 25 mg prostaglandin F₂α (Lutalyse, Upjohn, Kalamazoo, MI, USA) 17, 18 or 19 days after the last day of feeding MGA (MGA/PG); (2) a single injection of 25 mg of prostaglandin F₂α (PG); (3) 20 or 25 mg of prostaglandin F₂α injected i.m. at 12-day intervals (PG/PG) or (4) 50 or 100 µg of GnRH injected i.m., followed by 25 mg of prostaglandin F₂α 7 days later (GnRH/PG).

Heifers were inspected visually for standing estrus mornings and evenings, but inseminated only in the evenings after 16:00, approximately ½ or 1 day after onset of estrus. Insemination was either into the uterine body conventionally, or half into each uterine horn using atraumatic embryo transfer sheaths (IMV, Minneapolis, MN, USA). In the latter case, semen was deposited past the greater curvature of the uterine horn as far anterior as could be accomplished without trauma, identically to nonsurgical embryo transfer. In most cases, semen was deposited between the anterior third and mid-cornua.

Most experiments included a frozen sperm control inseminated into the uterine body with 20 or 40 x 10⁶ sperm/dose from the same bulls used for sperm sorted for sex-type ("sexed"). This control served as a composite estimate of the intrinsic, normal fertility of the heifers under the specific field-trial conditions as well as the fertility of the bulls used and the skills of the inseminators. Some trials also included a low-dose, unsexed control group. Sometimes numbers of control inseminations were planned to be ½ or ⅔ the number used for each treatment to obtain more information on sexed sperm. Frozen sexed and control sperm were thawed for 20 to 30 sec in a 35 to 37°C water bath. Various other details are summarized in Table 9.

Pregnancy was diagnosed by ultrasound 28 to 37 d post insemination and/or 56 to 92 d post-insemination, at which time fetal sex was determined in most trials, as described in Curran, S., Theriogenology 1991; 36:809-814, without the operator's knowing insemination treatments or controls. Sexes of calves born were nearly identical to the fetal-sex diagnosis. Data were

analyzed by single-degree-of-freedom Chi square corrected for continuity; 2-tail tests were used unless 1-tail is specified. Fewer than 5% of the inseminations were culled due to errors of insemination treatment, frank infection of the reproductive tract, failure to traverse the cervix, etc. Decisions to cull animals from experiments were made shortly after insemination and were never based on the pregnancy diagnosis.

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Table 9. Procedural details of field trials

	Trial	Insemination dates	Breeds of heifers	Bulls used	Inseminator s	Estrus synchroni- zation	Comments
5	1	5/20-23, 1997	Angus	N1, N2, AN4	A, B	MGA/PG	Included low-dose controls
	2	2/18-5/22, 1998	Angus crossbred	N3, N4, N5, N6	C, D	PG/PG	Low dose but no normal- dose controls; some heifers pregnant and aborted when synchronized
10	3	6/2-6/5, 1998	Angus	AN4, AN5, N7, N8	B, D	MGA/PG	
	4	2/10-13, 1999	Holstein	J2, J4	C, D	PG	Very severe mud, snow, wind, and cold, driving rain
15	5	2/24-26, 1999	Holstein	J2, J4, J5	C, B, D	PG/PG	
	6	4/14-16, 1999	Holstein	J2, J3, J4, J5	C, D	PG	Some heifers were reproductive culls
20	7	4/27-5/1, 1999	Hereford& Angus crossbred	AN1, AN4	C	MGA/PG	Semen for 1 bull shipped 6 h before sorting; severe weather
	8	4/21-23, 1999	Angus crossbred	H1, H2	E	MGA/PG	Feedlot heifers
25	9	5/5-8, 1999	Red Angus	AR1, AR2	C, F	MGA/PG	
	10	5/31-6/2, 1999	Angus	AN4, AN7, AN8	B, D	GnRH/ PG	
	11	7/28-30, 1999	Holstein	H2, H3	C, D	PG/PG	First replicate available in a much larger trial
30							

RESULTS AND DISCUSSION

The data presented are from 11 consecutive, heterogeneous field trials, constrained by logistical aspects of the studies, such as having to match bulls to genetic needs of the herds, unavailability of fertility information on bulls, limited numbers of heifers, unavailability of the same inseminators across trials, severe weather in some trials, limited amounts of sexed semen in early trials, 2 sets of heifers in which some turned out to be pregnant up to about 55 days at the time of estrus synchronization, etc. Up to 4 bulls and 3 inseminators were involved with each trial; this enabled us to sample populations to ensure that results applied to more than one bull or technician; however, insufficient data were produced to evaluate bull-to-bull differences in fertility rigorously.

Most sets of heifers were from breeding herds located 140 to 250 km from our laboratory. There were no significant differences in pregnancy rates between inseminators in any trial, but numbers of breedings per inseminator were low, and differences likely would be detected with larger numbers of inseminations.

Estrus synchronization methods were not compared within trials, so it was not possible to compare pregnancy rates among these methods. Pregnancy rates appeared to be satisfactory for all four synchronization procedures used.

Since inseminations were done once a day, heifers in estrus evenings were inseminated approximately 24 h after estrus was detected. The pregnancy rate for these heifers with sexed sperm pooled over all trials was 203/414 (49.0%), which was not significantly different ($P>0.1$) from that of heifers in estrus mornings and thus inseminated half a day after estrus detection 266/586 (45.4%). This tendency for higher fertility with later insemination is in agreement with findings from other research that it is preferable to inseminate later than normally recommended with lower fertility bulls, when low sperm numbers are used, or when conditions are otherwise suboptimal.

Pregnancy rates by treatments and, when available, fetal or calf sex are presented in Tables 10 to 20. The objective was to obtain female offspring, except in trial 8; accuracy was 95%, 83%, 90%, 83%, 82%, and 94% in Trials 1, 3, 8, 9, 10, and 11, respectively. In the remainder of the trials, fetal or birth sexes were not available because of timing of pregnancy diagnosis, unavailability of persons skilled in sexing fetuses, and/or because calves have not yet

been born. This was not a major concern because the main objective of this research was to determine fertility of flow-sorted sperm inseminated at low doses.

The accuracy of sexing can be adjusted to virtually any level desired between 50 and 95+% by adjusting the sorting parameters. However, higher accuracy results in lower numbers of sperm sorted per unit time, particularly for Y-chromosome sperm. 90% accuracy is sufficient for routine work.

The main findings from each field trial will be summarized in turn. Note that total sperm numbers are given in table headings; numbers of progressively motile sperm usually were 30 to 50% of these values. Field trial 1 (Table 10) confirmed that pregnancy rates with uterine horn insemination using low numbers of unsexed sperm were similar to controls with normal sperm numbers. The day 64 to 67 pregnancy rate with unfrozen sexed sperm (42%) was 12 percentage points below the unsexed liquid control with sperm diluted, stained, and centrifuged identically to the sorted sperm. Accuracy of sexing was 95%; the sex of calves born from sexed sperm matched the sex diagnosis of fetuses exactly; there was one mistake in sexing fetuses of controls. There were no abortions between 2 months of gestation and term, and all 19 calves from the sexed sperm treatment were normal and survived. For the sexed semen treatment, the 2-month pregnancy rates for bulls N1, N2, and N3 were 41, 44, and 40%, respectively; 39% (13/33) of heifers in estrus in the morning and 50% (6/12) in estrus in the evening became pregnant.

Table 10. Results of field trial 1 -- Angus heifers in Wyoming, 1997

Treatment/site	No. sperm	No. heifers	No. pregnant day 31 to 33	No. pregnant day 64 to 67	No. ♀ calves
Sexed, 5°C/horns	3 x 10 ⁵	45	20 (44%)	19 (42%)	18 (95%) ^a
Control, 5°C/horns	3 x 10 ⁵	28	15 (54%)	15 (54%)	5 (53%) ^b
Frozen, control/body	40 x 10 ⁶	29	16 (55%)	15 (52%)	11 (73%) ^{a,b}

^{a,b} Sex ratios without common superscripts differ (P<0.02).

Field trial 2 (Table 11) provided the first evidence that results with sexed, frozen sperm are similar to sexed, unfrozen sperm if adjustment is made for numbers of sperm killed during cryopreservation. There also was no difference in pregnancy rates between sexed sperm stored at 5 versus 18°C. Pregnancy rates at 2+ months after insemination for sexed semen from individual bulls ranged from 22 to 42% pregnant (P>0.05). Embryonic loss between 1 and 2 months of gestation was very similar for sexed and control pregnancies. Calving data were available from 39 heifers from this trial; each of these heifers (30 sexed pregnancies, 9 controls) pregnant at 2 months calved after a normal-length gestation.

Table 11. Results of field trial 2--Crossbred beef heifers in Colorado, 1998

Treatment/site	No. sperm	No. heifers	No. pregnant day 30 to 35 ^a	No. pregnant day 59 to 92 ^a
Control, 5°C/horns	5 x 10 ⁵	58	27 (47%)	24 (41%)
Sexed, 5°C/horns	5 x 10 ⁵	51	17 (33%)	16 (31%)
Sexed, 18°C/horns	5 x 10 ⁵	46	16 (35%)	12 (26%)
Sexed, frozen/horns	1 x 10 ⁶	87	29 (33%)	28 (32%)

^a No significant differences, χ^2 .

Field trial 3 (Table 12) confirmed that sexed, frozen sperm results in reasonable pregnancy rates. The accuracy of sexing sperm was confirmed again; however, there were 4 errors in sexing fetuses relative to the calves born; the actual sexes of calves born are presented. Again, there were no abortions between 2 months of gestation and term. Pregnancy rates averaged over sexed, unfrozen and sexed, frozen sperm for bulls N8, N9, AN5, and AN4 were 24, 31, 50, and 60%, respectively (P<0.1).

Table 12. Results of field trial 3 -- Angus heifers in Wyoming, 1998

Treatment/site	No. sperm	No. heifers	No. pregnant day 62 to 65	No. ♀ calves
Sexed, 18°C/horns	5 x 10 ⁵	37	11 (30%) ^a	10 (91%) ^c
Sexed, frozen/horns	1 x 10 ⁶	35	18 (51%) ^{a,b}	14 (78%) ^c
Frozen, control/body	40 x 10 ⁶	37	27 (73%) ^b	16 (59%) ^d

^{a,b} Means without common superscripts differ P<0.05).

^{c,d} The percentage of ♀ calves from the sexed treatments (83%) differed from the control group, P<0.05, 1-tail, χ^2 .

Field trials 4, 5, and 6 (Tables 13, 14, 15) were done at the same location with 3 different groups of heifers. Unfortunately, it was not possible to replicate each trial similarly due to vagaries of field trials, such as scheduling personnel, availability of sexed semen from each bull, etc. The widely different pregnancy rates between trials 5 and 6 illustrate that conditions were different among trials. Some of the heifers in trial 6 were available because they failed to get pregnant after a month of natural mating. Under conditions of these trials, pregnancy rates were very similar between 1.5 and 3.0 x 10⁶ sexed, frozen sperm/dose. Furthermore, there was no advantage to uterine-horn insemination. There were no significant differences (P>0.05) in pregnancy rates among bulls except in Trial 5 in which the pregnancy rate of J2, 20/28 (71%), was higher than that of J4, 15/39 (38%) (P<0.05). This difference was not consistent from trial to trial, as J4 had numerically but not significantly (P>0.1) higher pregnancy rates than J2 in Trials 4 and 6.

Table 13. Results of field trial 4 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 30 to 33	No. pregnant day 64 to 67*
Sexed, frozen/body	1.5 x 10 ⁶	55	36 (65%) ^{a,b}	36 (65%) ^{a,b}
Sexed, frozen/body	3 x 10 ⁶	52	27 (52%) ^a	26 (50%) ^a
Control, frozen/body	20 x 10 ⁶	55	45 (82%) ^b	43 (78%) ^b

^{a,b} Means without common superscripts differ (P<0.01).

* Six heifers pregnant at d 30 to 33 were sold before the second pregnancy diagnosis; these were assumed to have remained pregnant.

Table 14. Results of field trial 5 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 33 to 35 ^a	No. pregnant day 60 to 62 ^a
Sexed, frozen/body	1.5 x 10 ⁶	23	12 (52%)	12 (52%)
Sexed, frozen/body	3.0 x 10 ⁶	25	15 (60%)	14 (56%)
Sexed, frozen/horns	1.5 x 10 ⁶	25	15 (60%)	12 (48%)
Sexed, frozen/horns	3.0 x 10 ⁶	25	17 (68%)	15 (60%)
Control, frozen/body	20 x 10 ⁶	30	20 (67%)	19 (63%)

^a No significant differences.

Table 15. Results of field trial 6 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 31 to 34	No. pregnant day 60 to 63
Sexed, frozen/body	1.5 x 10 ⁶	27	11 (41%) ^a	9 (33%) ^a
Sexed, frozen/body	3.0 x 10 ⁶	25	10 (40%) ^a	9 (36%) ^a
Sexed, frozen/horns	1.5 x 10 ⁶	24	8 (33%) ^a	7 (29%) ^a
Sexed, frozen/horns	3.0 x 10 ⁶	24	10 (42%) ^a	8 (33%) ^a
Control, frozen/body	20 x 10 ⁶	24	18 (75%) ^b	17 (71%) ^b

^{a,b} Means without common superscripts differ (P<0.05).

For trial 7 (Table 16), only one inseminator was available due to rescheduling. This is the only trial that showed a convincing advantage of uterine-horn over uterine-body insemination. For this inseminator under the conditions of the trial, 55% more heifers (22 percentage points) became pregnant with sexed, frozen semen inseminated into the uterine horns than into the uterine body. The true difference could be smaller because there are wide confidence intervals on these means. In all the other trials (5, 6, 9, and 11) in which body- and horn-insemination were compared, pregnancy rates were very similar for both methods for this technician as well as for other technicians.

Semen from one of the bulls used in Trial 7 was shipped without dilution from Montana by air in an insulated box at ~20°C before sorting; shipping time was 6 h. Pregnancy rates for the sexed sperm from the two bulls were virtually identical, 49% for the unshipped and 52% for the shipped semen. Semen was not diluted with extender and not cooled for shipping because staining properties of sperm with Hoechst 33342 are altered by dilution with extenders. Furthermore, in other studies (see Example 4), storing semen neat at ambient temperature between collection and flow-sorting was found to be superior to diluting it.

Table 16. Results of field trial 7 -- Crossbred beef heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 33 to 37
Sexed, frozen/body	1.5 x 10 ⁶	86	34 (40%) ^a
Sexed, frozen/horns	1.5 x 10 ⁶	86	53 (62%) ^b
Control, frozen/body	20 x 10 ⁶	35	18 (51%) ^{a,b}

^{a,b} Means without common superscripts differ (P<0.01).

Field trial 8 (Table 17) concerned feedlot heifers not implanted with growth promotants; at the time pregnancy was diagnosed they were aborted, so calving data was not available. This experiment illustrates that efficacious sexing also can be done in the male direction. Pregnancy rates for the 2 bulls were 50 and 61%.

Table 17. Results of field trial 8 -- Angus heifers in Nebraska, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant ^a day 74 to 76	No. ♂ fetuses
Sexed, frozen, 72 mW laser/body	1 x 10 ⁶	18	7 (39%)	6 (86%)
Sexed, frozen, 135 mW laser/body	1 x 10 ⁶	18	13 (78%)	12 (92%)

^a No significant differences.

Field trial 9 (Table 18) was the only trial to show a convincing advantage of 3.0 versus 1.5 x 10⁶ sexed, frozen sperm/insemination dose. This advantage was true for both inseminators. Pregnancy rates for sexed sperm from the 2 bulls were 62 and 75%.

Table 18. Results of field trial 9 -- Red Angus heifers in Nebraska, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 60 to 63 ^a	No. ♀ fetuses
Sexed, frozen/body	1.5 x 10 ⁶	15	8 (53%)	7 (88%)
Sexed, frozen/body	3.0 x 10 ⁶	14	12 (86%)	9 (75%)
Sexed, frozen/horns	1.5 x 10 ⁶	16	9 (56%)	7 (78%)
Sexed, frozen/horns	3.0 x 10 ⁶	16	12 (75%)	11 (92%)
Control, frozen/body	20 x 10 ⁶	30	21 (70%)	13 (62%)

^a 3.0 x 10⁶ sexed sperm had a higher pregnancy rate (80%) than 1.5 x 10⁶ sexed sperm (55%), P<0.05, 1-tail χ^2 .

Pregnancy rates in field trial 10 (Table 19) with sexed, frozen semen, were similar to controls; the accuracy of sexing sperm on this trial was only 82%, which, however, is not significantly different from the targeted 90% accuracy. Pregnancy rates for sexed semen were 54, 66, and 50% for bulls AN4, AN7, and AN8, respectively (P>0.1). Eighteen of the heifers inseminated in this trial were the calves resulting from sexed sperm in field trial 1.

Table 19. Results of field trial 10 -- Angus heifers in Wyoming, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 61 to 63 ^a	No. ♀ fetuses
Sexed, frozen/body	1 x 10 ⁶	44	26 (59%)	23 (85%)
Sexed, frozen/body	3 x 10 ⁶	43	23 (53%)	17 (74%)
Control, frozen/body	20 x 10 ⁶	35	20 (57%)	12 (57%)

^a No significant differences.

Table 20. Results of field trial 11 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 28 to 30 ^a	No. pregnant day 56 to 58 ^{a,b}
Sexed, frozen/body	1 x 10 ⁶	12	8 (67%)	7 (58%)
Sexed, frozen/body	3 x 10 ⁶	12	6 (50%)	4 (33%)
Sexed, frozen/horns	1 x 10 ⁶	7	4 (57%)	4 (57%)
Sexed, frozen/horns	3 x 10 ⁶	7	4 (57%)	4 (57%)
Control, frozen/body	20 x 10 ⁶	9	4 (44%)	3 (33%)

^a No significant differences, χ^2 .

^b 16 of 17 (94%) fetuses from the sexed semen treatments were female; 2 were too deep in the body cavity to sex with ultrasound.

Data from trials were combined in which treatments were identical except 1 x 10 and 1.5 x 10⁶ sperm doses were pooled (Table 21).

Table 21. Meta-summary from combining trials with sexed, frozen semen and frozen controls.

Trials combined	Sperm no./site	No. heifers	No. pregnant
5, 6, 9, 11	1.0 - 1.5 x 10 ⁶ /body	77	36 (47%)
	3.0 x 10 ⁶ /body	76	38 (50%)
	1.0 - 1.5 x 10 ⁶ /horns	72	32 (44%)
	3.0 x 10 ⁶ /horns	72	39 (54%)
	20 x 10 ⁶ /body, control	93	61 (66%)
4, 5, 6, 9, 10, 11	1.0 - 1.5 x 10 ⁶ /body	176	98 (56%)
	3.0 x 10 ⁶ /body	171	88 (51%)
	20 x 10 ⁶ /body, control	183	124 (68%)
5, 6, 7, 9, 11	1.5 x 10 ⁶ /body	163	70 (43%)
	1.5 x 10 ⁶ /horn	158	85 (54%)
	20 x 10 ⁶ /body, control	128	79 (62%)

Pregnancy rates with sexed sperm were generally 70-90% of unsexed controls within experiments with 7 to 20 times more sperm. This difference was less in the more recent trials, possibly reflecting improved sexing and sperm-processing procedures.

In some trials, heifers were examined for pregnancy by ultrasound at both 1 and 2 months after insemination. Pregnancy losses in this interval were similar ($P>0.1$) for sexed (23/261; 8.8%) versus control (9/145; 6.2%) sperm treatments, which is one measure that genetic damage due to sexing is minimal. Calving information was obtained from only a few of the pregnant heifers because most cattle from the earlier trials were sold, and those from later trials have not calved yet. The population of calves produced to date from sexed semen appears to be no different from the population of controls.

CONCLUSION

Sex ratios in cattle can be distorted to about 90% of either sex by sorting sperm on the basis of DNA content with a flow cytometer/cell sorter followed by cryopreservation and relatively routine artificial insemination. Calves resulting from sexed sperm appear to be normal. For most bulls in these studies, pregnancy rates with 1.0 to 1.5×10^6 sexed, frozen sperm were 70 to 90% of unsexed controls with 20 or 40 $\times 10^6$ frozen sperm inseminated conventionally. These results apply to well-managed heifers bred by well-trained inseminators using properly processed semen. There may be a small advantage to inseminating sexed sperm bilaterally into the uterine horns compared to standard uterine body insemination.

The present invention has of necessity been discussed herein by reference to certain specific methods and materials. It is to be understood that the discussion of these specific methods and materials in no way constitutes any limitation on the scope of the present invention, which extends to any and all alternative materials and methods suitable for accomplishing the ends of the present invention.

All patents and publications described are herein incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

~~1 1. A method for the cryopreservation of sperm comprising:~~

- ~~2 (a) obtaining a selected sperm sample;~~
- ~~3 (b) cooling said selected sperm sample;~~
- ~~4 (c) isolating sperm from said selected sperm sample to produce isolated sperm;~~
- ~~5 (d) adding final extender to said isolated sperm to produce a suspension of~~
- ~~6 sperm; and~~
- ~~7 (e) freezing said suspension of sperm.~~

~~1 2. The method of Claim 1 wherein said selected sperm sample comprises a portion of the~~

~~2 sperm present in a source sample, said portion of sperm selected for a characteristic, and~~

~~3 wherein the sperm concentration in the selected sperm sample is lower than in the source~~

~~4 sample.~~

~~1 3. The method of Claim 1 wherein said selected sperm sample comprises sex-selected~~

~~2 sperm.~~

~~1 4. The method of Claim 1 wherein said selected sperm sample comprises mammalian~~

~~2 sperm.~~

~~1 5. The method of Claim 4 wherein said selected sperm sample comprises bovine sperm.~~

~~1 6. The method of Claim 4 wherein said selected sperm sample comprises equine sperm.~~

~~1 7. The method of Claim 4 wherein said selected sperm sample comprises porcine sperm.~~

~~1 8. The method of Claim 1 wherein said selected sperm sample comprises sperm selected by~~

~~2 a method from the group consisting of flow cytometry, a magnetic technique, a columnar~~

~~3 technique, a gravimetric technique, a biochemical technique, a technique based on~~

4 motility of sperm, a technique based on an electrical property of sperm, and any
5 combination thereof.

1 9. The method of Claim 8 wherein said sperm have been selected by flow cytometry.

1 10. The method of Claim 1 wherein cooling is carried out by reducing the temperature of the
2 selected sperm sample to about 5°Celsius.

1 11. The method of Claim 10 wherein cooling is carried out over a period of about 60 minutes
2 to about 240 minutes.

1 12. The method of Claim 1 wherein the final extender added to said selected sperm sample
2 each comprise, in addition to a cryoprotectant, one or more of the following components:
3 a component that maintains osmolality and buffers pH, an organic substance that reduces
4 cold shock and preserves fertility of sperm, an energy source, a substance that facilitates
5 sperm capacitation, and an antibiotic.

1 13. The method of Claim 12 wherein said cryoprotectant is selected from the group
2 consisting of disaccharides, trisaccharides, and any combination thereof.

1 14. The method of Claim 12 wherein said cryoprotectant is selected from the group
2 consisting of glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any
3 combination thereof.

1 15. The method of Claim 12 wherein said component that maintains osmolality and buffers
2 pH is selected from the group consisting of a buffer comprising a salt, a buffer containing
3 a carbohydrate, and any combination thereof.

1 16. The method of Claim 12 wherein said component that maintains osmolality and buffers
2 pH is selected from the group consisting of sodium citrate,

3 ~~Tris[hydroxymethyl]aminomethane,~~

4 ~~N-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, monosodium glutamate,~~
5 milk, HEPES buffered medium, and any combination thereof.

1 17. The method of Claim 12 wherein said organic substance is selected from the group
2 consisting of egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin,
3 and any combination thereof.

1 18. The method of Claim 12 wherein said energy source is a monosaccharide selected from
2 the group consisting of glucose, fructose, mannose, and any combination thereof.

1 19. The method of Claim 12 wherein said antibiotic is selected from the group consisting of
2 tylosin, gentamicin, lincomycin, linco-spectin, spectinomycin, penicillin, streptomycin,
3 and any combination thereof.

1 20. The method of Claim 1 wherein, after the addition of the final extender, the sperm sample
2 and suspension of sperm, respectively, comprise glycerol, sodium citrate,
3 Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.

1 21. The method of Claim 1 wherein, after the addition of the final extender, said sperm
2 sample and suspension of sperm, each comprise glycerol, sodium citrate, egg yolk, and
3 one or more antibiotics.

1 22. The method of Claim 1 wherein, after the addition of the final extender, said sperm
2 sample and suspension of sperm, each comprise glycerol, egg yolk, milk, fructose, and
3 one or more antibiotics.

1 23. The method of Claim 1 wherein said extender has a pH in the range of about 6.5 to about
2 7.5.

1 24. The method of Claim 1 wherein the sperm are isolated from said selected sperm sample
2 by centrifugation.

~~1 25. The method of Claim 24 wherein said centrifugation allows for at least about 50% to
2 about 90% recovery of sperm.~~

~~1 26. The method of Claim 1 wherein the concentration of sperm in said suspension prior to
2 freezing is about 1×10^6 /ml to about 300×10^6 /ml.~~

✓ 1 27. A frozen selected sperm sample comprising a portion of the sperm present in a source
2 sample, said portion of sperm selected for a characteristic.

1 28. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample
2 comprises sex-selected sperm.

1 29. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample
2 comprises mammalian sperm.

1 30. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample
2 comprises bovine sperm.

1 31. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample
2 comprises equine sperm.

1 32. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample
2 comprises porcine sperm.

1 33. The frozen selected sperm sample of Claim 27 wherein the method used to select said
2 selected sperm sample comprises a technique from the group consisting of flow
3 cytometry, a magnetic technique, a columnar technique, a gravimetric technique, a
4 biochemical technique, a technique based on motility of sperm, a technique based on an
5 electrical property of sperm, and any combination thereof.

1 34. The frozen selected sperm sample of Claim 33 wherein said frozen selected sperm sample
2 comprises sperm that have been selected by flow cytometry.

1 35. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample
2 is produced by a method comprising:

- 3 (a) obtaining a selected sperm sample;
4 (b) cooling said selected sperm sample;
5 (c) isolating sperm from said selected sperm sample to produce isolated sperm;
6 (d) adding final extender to said isolated sperm to produce a suspension of
7 sperm; and
8 (e) freezing said suspension of sperm.

1 36. A method comprising using the frozen selected sperm sample of Claim 27 for artificial
2 insemination or in vitro fertilization.

1 37. The method of Claim 36 comprising using said frozen selected sperm sample for low-
2 dose artificial insemination.

add B1

DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION

As a below named inventor,

I hereby declare that my residence, post office address and citizenship is as stated below next to my name.

I believe I am the original and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: "METHOD OF CRYOPRESERVING SELECTED SPERM CELLS", the specification of which

 X is attached hereto.

 was filed on and assigned
Application Serial Number

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign applications for patent or inventor's certificate, listed below and so identified, and I have also identified below any foreign application for patent or inventor's certificate on this invention filed by me or my legal representatives or assigns and having a filing date before that of the application on which priority is claimed.

Number	Country	Day/Month/ Year Filed	Priority Claimed - Yes or No

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States applications listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

Application Serial No.	Filing Date	Status

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States provisional application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior provisional application and the filing date of this application.

Provisional Application No.	Filing Date	Status
60/167,423	November 24, 1999	Pending

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following attorneys and agents, with full power of substitution and revocation, to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith and request that all correspondence and telephone calls with respect to this application be directed to McCUTCHEN, DOYLE, BROWN & ENERSEN, LLP Three Embarcadero Center, San Francisco, California 94111, Telephone No. (415) 393-2000:

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Inventor's signature: John L. Schenk

Date: 21 Dec 99

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John L. Schenk
Application No.: Unknown
Filed: December 17, 1999
For: METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 C.F.R. §§ 1.9(f) AND 1.27(c)) - SMALL BUSINESS CONCERN**

I hereby declare that I am

- ☐ the owner of the small business concern identified below:
☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: XY, Inc.
ADDRESS: 3801 W. Rampart Road
ARBL Building, CSU Foothills Research Campus
Fort Collins, CO 80523

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 C.F.R. § 121.3-18, for purposes of paying reduced fees under § 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled METHOD OF CRYOPRESERVING SELECTED SPERM CELLS by inventor, John L. Schenk

described in

- (X) the specification filed herewith
() application Serial No. _____, filed _____
() Patent No. [_____], issued [Date]

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below¹ and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 C.F.R. § 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

Full Name: _____

Address: _____

() Individual () Small Business Concern () Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 C.F.R. § 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of person signing: DR. MERVYN JACOBSON

Title of person
other than owner: PRESIDENT & CEO.

Address of person signing: XY, Inc.
3801 W. Rampart Road
ARBL Building, CSU Foothills Research Campus
Fort Collins, CO 80523

Signature: [Signature]

Date: Dec 23rd 1999.

¹NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 C.F.R. § 1.27)

Please type a plus sign (+) inside this box.



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UTILITY PATENT APPLICATION TRANSMITTAL

Attorney Docket No

22091-701 DIV2

Total Pages

78

First Named Inventor or Application Identifier

John Schenk

Express Mail Label No.

EL548626415US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents:

Assistant Commissioner for Patents
ADDRESS TO: Box Patent Application
Washington, DC 20231

1. ☒ Fee Transmittal Form
(Submit an original, and a duplicate for fee processing)

6. ☐ Microfiche Computer Program (Appendix)

2. ☒ Specification [Total Pages 61]
(preferred arrangement set forth below)

7. Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)

- Descriptive title of the invention
- Cross References to Related Applications
- Statement Regarding Fed sponsored R & D
- Reference to Microfiche Appendix
- Background of the invention
- Brief Summary of the Invention
- Brief Description of the Drawings (if filed)
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

a. ☐ Computer Readable Copy

b. ☐ Paper Copy (identical to computer copy)

c. ☐ Statement verifying identity of above copies

3. ☐ Drawings (35 USC 113) [Total Sheets ☐

4. Oath or Declaration [Total Pages ☐

a. ☐ Newly executed (original or copy)

b. ☒ Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)

[Note Box 5 below]

i. ☐ DELETION OF INVENTOR(S)

Signed statement attached deleting inventor(s) named in
the prior application, see 37 CFR 1.63(d)(2) and 1.33(b)

5. ☒ Incorporated By Reference (useable if Box 4b is checked) The entire
disclosure of the prior application, from which a
copy of the oath or declaration is supplied under Box 4b,
is considered as being part of the disclosure of the accompanying
application and is hereby incorporated by reference therein.

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))

9. ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney
(when there is an assignee)

10. ☐ English Translation Document (if applicable)

11. ☒ Information Disclosure ☐ Copies of IDS
Statement (IDS)/PTO-1449 Citations

12. ☒ Preliminary Amendment

13. ☒ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)

14. ☒ Small Entity ☒ Statement filed in prior application,
Statement(s) Status still proper and desired

15. ☐ Certified Copy of Priority Document (s)
(if foreign priority is claimed)

16. ☐ Other:

17. If a CONTINUING APPLICATION,
check appropriate box and supply the requisite information:

☐ Continuation ☒ Divisional ☐ Continuation-in-part
(CIP)

of prior application No: 09/478,299 (filed January 5, 2000), which claims
the benefit of 60/167,423 (filed November 24, 1999)

18. CORRESPONDENCE ADDRESS



Customer Number or Bar Code Label

(Insert Customer No. or Attach Bar code label here)

or

☒

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#2
10-18-00

In The United States Patent and Trademark Office

In re Application of:	Group Art Unit: Not yet assigned
Assignee: XY, Inc.	Examiner: Not yet assigned
Inventors: John L. Schenk	Express Mail Label No.: EL548626415US
Serial No: Not yet assigned	Attorney Docket No.: 22091-701 DIV2
Filed: Herewith	
For: METHOD OF CRYOPRESERVING SELECTED SPERM CELLS	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to calculation of the claim fees, please amend the above-referenced patent application as follows:

IN THE SPECIFICATION

✓ At page 1, line 5, delete the following: "The applications".

At page 1, line 5, please insert the following [-This application is a divisional of
U.S. Patent Application No. 09/478,299, filed January 5, 2000, which--

IN THE CLAIMS

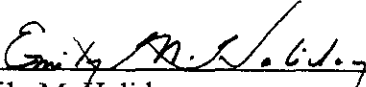
Cancel Claims 1-26.

CONCLUSION

No new matter has been added by these amendments. It is respectfully requested that the Examiner contact the undersigned attorney at (650) 849-4910 with any questions regarding the application.

Dated: May 24, 2000

Respectfully submitted,

By: 
Emily M. Haliday
Registration No.: 38,903

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center
San Francisco, California 94111
Telephone: (650) 849-4910
Telefax: (650) 849-4800

In The United States Patent and Trademark Office

JC815 U.S. PTO
09/577246
05/24/00

In re Application of:	Group Art Unit: Not yet assigned
Assignee: XY, Inc.	Examiner: Not yet assigned
Inventors: John Schenk	Express Mail Label No.: EL548626415US
Serial No: Not yet assigned	Attorney Docket No.: 22091-701 DIV2
Filed: Herewith	
For: METHOD OF CRYOPRESERVING SELECTED SPERM CELLS	

INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In accordance with 37 C.F.R. § 1.56, the references listed below and on the attached Form PTO-1449 are being brought to the attention of the Examiner for consideration in connection with the examination of the above-identified patent application.

1. United States Patent No. 4,267,268, Issued May 12, 1981;
2. United States Patent No. 4,980,277, Issued December 25, 1990;
3. United States Patent No. 5,135,759, Issued August 4, 1992;
4. United States Patent No. 5,514,537, Issued May 7, 1996;
5. International Publication No. WO 99/33956, Publication Date July 8, 1999;
6. Amann, Rupert P., "Cryopreservation of Sperm," *Encyclopedia of Reproduction*, 1:773-783 (1999);
7. Arriola, J. and Foote, R. H., "Glycerolation and Thawing Effects on Bull Spermatozoa Frozen in Detergent-Treated Egg Yolk and Whole Egg Extenders," *J Dairy Sci*, 70:1664-1670 (1987);

8. Behrman, S. J. and Ackerman, D. R., "Freeze Preservation of Human Sperm," *Am. J. Obstet. and Gynecol.*, 103:5:654-664 (1969);
9. Foote, R. H., "Buffers and Extenders: What Do They Do? Why Are They Important?" *Proc of the 10th NAAB Tech. Conf. on Artificial Insemination and Reproduction*, 62-70 (1984);
10. Graham, J. K. and Hammerstedt, R. H., "Differential Effects of Butylated Hydroxytoluene Analogs on Bull Sperm Subjected to Cold-Induced Membrane Stress," *Cryobiology*, 29:106-117 (1992);
11. Hammerstedt, et al, "Cryopreservation of Mammalian Sperm: What We Ask Them to Survive," *Journal of Andrology*, 11:1:73-88 (1990);
12. Maxwell, W.M.C., and Johnson, L. A., "Chlortetracycline Analysis of Boar Spermatozoa After Incubation, Flow Cytometric Sorting, Cooling, or Cryopreservation," *Molecular Reproduction and Development*, 46:408-418 (1997);
13. Polge, et al, "Revival of Spermatozoa After Vitriification and Dehydration at Low Temperatures," *Nature*, 164:666 (1949);
14. Pursel, et al, "Effect of Orvus ES Paste on Acrosome Morphology, Motility and Fertilizing Capacity of Frozen-Thawed Boar Sperm," *Journal of Animal Science*, 47:1:198-202 (1978);
15. Salisbury, et al, *Physiology of Reproduction and Artificial Insemination of Cattle*, 2nd Ed., San Francisco: W. H. Freeman, 442-554 (1978);
16. Schenk, et al, "Cryopreservation of Flow-Sorted Bovine Spermatozoa," *Theriogenology*, 52:1375-1391 (1999);

17. Seidel, et al, "Insemination of Heifers with Sexed Sperm," *Theriogenology*, 52:1407-1420 (1999);

18. Watson, P. F., "Recent Developments and Concepts in the Cryopreservation of Spermatozoa and the Assessment of Their Post-Thawing Function," *Reprod. Fertil. Dev.*, 7:871-891 (1995).

I. Timing of the Information Disclosure Statement:

This Information Disclosure Statement:

☒ Accompanies the new patent application submitted herewith. 37 CFR § 1.97(a).

☐ Is filed within three months after the filing date of the application or within three months after the date of entry of the national stage of a PCT application as set forth in 37 CFR § 1.491.

☐ As far as is known to the undersigned, is filed before the mailing date of a first Office Action on the merits. In the event, however, that an Office Action has crossed in the mail with this Information Disclosure Statement, the Commissioner is hereby authorized to charge Deposit Account No. 50-1189 for any fees required pursuant to 37 CFR §§ 1.17(p) or 1.17(i)(1).

☐ Is filed after the first Office Action and more than three months after the application's filing date or PCT national stage date of entry filing but, as far as is known to the undersigned, prior to the mailing date of either a final rejection or a notice of allowance and is accompanied by either the fee (\$240) set forth in 37 CFR § 1.17(p) or a certification as specified in 37 CFR § 1.97(e), as checked below.

Information Disclosure Statement -- Section 9 PTO-1449 (Modified) [6-1] Page 3 of 6

52023046.1
Docket No.: 22091-701 DIV2

☐ Is filed after the mailing date of either a final rejection or a notice of allowance, whichever occurred first, and is accompanied by the fee (\$130) set forth in 37 CFR § 1.17(i)(1) and a certification as specified in 37 CFR § 1.97(e), as checked below. This document is to be considered as a petition requesting consideration of the Information Disclosure statement.

The undersigned certifies that:

☐ Each item of information contained in the Information Disclosure Statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this Information Disclosure Statement.

☐ No item of information contained in this Information Disclosure Statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application or, to the knowledge of the undersigned after making reasonable inquiry, was known to any individual designated in 37 CFR § 1.56(c) more than three months prior to the filing of this Information Disclosure Statement.

II. Copies of the Cited Items:

☐ Copies of all of the items listed on the attached Form PTO-1449 are enclosed.

☐ Copies of only the following items listed on the attached Form PTO-1449 are enclosed:

☒ Copies of those items which are marked with an asterisk (*) in the attached Form PTO-1449 are not supplied because they were previously cited by or submitted to the Office in a prior Application No. 09/478,299, filed January 5, 2000, and relied upon in this application for an earlier filing date under 35 U.S.C. § 120. See 37 C.F.R. § 1.98(d).

☐ Copies of those items which are marked with a double asterisk (**) in the attached Form PTO-1449 are not supplied because they are U.S. patent application(s) and no copy of a U.S. patent application needs to be provided pursuant to 37 C.F.R. § 1.98(a)(2)(iii).

III. Concise Explanation of Relevance:

☐ A concise explanation of relevance of the items listed on Form PTO-1449 is not given.

☐ A concise explanation of relevance of [some of] the items listed on Form PTO-1449 is in the form of an English language copy of a Search Report from a foreign patent office, issued in a counterpart application, which refers to the relevant portions of the references [copy attached].

☐ A concise explanation of relevance of the items listed on form PTO-1449 is given for only non-English language listed item(s). Specifically, the following foreign-language documents are being brought to the attention of the Examiner along with a concise explanation of the relevance of each document, as it is presently understood by the individual designated in 37 C.F.R. § 1.56(c) most knowledgeable about the content of the information in each document:

IV. Conclusion:

Citation of the above documents shall not be construed as:

1. an admission that the documents are necessarily prior art with respect to the instant invention;
2. a representation that a search has been made, other than as described above; or
3. an admission that the information cited herein is, or is considered to be, material to patentability as defined in § 1.56(b).

Serial No.: 09/478,299

It is respectfully requested that the Examiner indicate consideration of the cited references by returning a copy of the attached form PTO 1449 with initials or other appropriate marks.

The Commissioner is hereby authorized to charge Deposit Account No. 50-1189 for any additional fees required in connection with the filing of this Information Disclosure Statement.

Please address all correspondence regarding this communication to the following address:

Emily M. Haliday
McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center
San Francisco, California 94111
Telephone: (650) 849-4910

Dated: May 24, 2000

Respectfully submitted,

By: Emily M. Haliday
Emily M. Haliday
Registration No. 38,903

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center
San Francisco, California 94111
Telephone: (650) 849-4910
Telefax: (650) 849-4800

Information Disclosure Statement -- Section 9 PTO-1449 (Modified) [6-1] Page 6 of 6

52023046.1
Docket No.: 22091-701 DIV2

FORM PTO-1449 (Modified) LIST OF PATENTS AND PUBLICATIONS FOR APPLICANT(S)' INFORMATION DISCLOSURE STATEMENT (Use several sheets if necessary)	ATTY. DOCKET NO. 22091-701 DIV2	SERIAL NO. Not yet assigned 09/577,246
	APPLICANT John L. Schenk	
	FILING DATE Herewith 5/24/00	GROUP ART UNIT Not yet assigned 1651

JCB15 U.S. PTO
 09/577246
 05/24/00

REFERENCE DESIGNATION U.S. PATENT DOCUMENTS

EXAM'R INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	Subclass	Filing Date If Appropriate
MM	A1 *	4,267,268	May 12, 1981	Nelson, Jr.	435	2	
	A2 *	4,980,277	December 25, 1990	Junnila	435	2	
U	A3 *	5,135,759	August 4, 1992	Johnson	424	561	
	A4 *	5,514,537	May 7, 1996	Chandler	435	2	

FOREIGN PATENT DOCUMENTS

EXAM'R INITIAL		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	Subclass	TRANSLATION	
							yes	no
MM	B1 *	WO 99/33956	July 8, 1999	PCT				

OTHER ART (Include Author, Title, Date, Pertinent Pages, etc.)

MM	C1 *	Amann, Rupert P.; "Cryopreservation of Sperm," <i>Encyclopedia of Reproduction</i> , 1:733-783 (1999).
	C2 *	Arriola, J. and Foote, R. H.; "Glycerolation and Thawing Effects on Bull Spermatozoa Frozen in Detergent-Treated Egg Yolk and Whole Egg Extenders," <i>J Dairy Sci</i> , 70:1664-1670 (1987).
	C3 *	Behrman, S. J. and Ackerman, D. R.; "Freeze Preservation of Human Sperm," <i>Am. J. Obstet. and Gynec.</i> , 103:5:654-664 (1969).
	C4 *	Foote, R. H., "Buffers and Extenders: What Do They Do? Why Are They Important?" <i>Proc of the 10th NAAB Tech. Conf. on Artificial Insemination and Reproduction</i> , 62-70 (1984).
	C5 *	Graham, J. K. and Hammerstedt, R. H.; "Differential Effects of Butylated Hydroxytoluene Analogs on Bull Sperm Subjected to Cold-Induced Membrane Stress," <i>Cryobiology</i> , 29:106-117 (1992).
	C6 *	Hammerstedt, et al, "Cryopreservation of Mammalian Sperm: What We Ask Them to Survive," <i>Journal of Andrology</i> , 11:1:73-88 (1990).
	C7 *	Maxwell, W. M. C. and Johnson, L. A.; "Chlortetracycline Analysis of Boar Spermatozoa After Incubation, Flow Cytometric Sorting, Cooling, or Cryopreservation," <i>Molecular Reproduction and Development</i> , 46:408-418 (1997).
U	C8 *	Polge, et al, "Revival of Spermatozoa After Vitrification and Dehydration at Low Temperatures," <i>Nature</i> , 164:666 (1949).

EXAMINER	DATE CONSIDERED
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EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant(s).


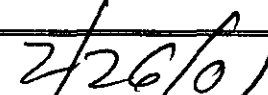
(Information Disclosure Statement -- Section 9 PTO-1449 (Modified) [6-1] Page 1 of 2

52023057.1

Docket No.: 22091-701DIV2

OTHER ART (Include Author, Title, Date, Pertinent Pages, etc.) [CONTINUED]

✓	C9 *	Pursel, et al, "Effect of Orvus ES Paste on Acrosome Morphology, Motility and Fertilizing Capacity of Frozen-Thawed Boar Sperm," <i>Journal of Animal Science</i> , 47:1:198-202 (1978).
✓	C10 *	Salisbury, et al, <i>Physiology of Reproduction and Artificial Insemination of Cattle</i> , 2nd Ed., San Francisco: W. H. Freeman, 442-554 (1978).
✓	C11 *	Schenk, et al, "Cryopreservation of Flow-Sorted Bovine Spermatozoa," <i>Theriogenology</i> , 52:1375-1391 (1999).
✓	C12 *	Seidel, et al, "Insemination of Heifers with Sexed Sperm," <i>Theriogenology</i> , 52:1407-1420 (1999).
✓	C13 *	Watson, "Recent Developments and Concepts in the Cryopreservation of Spermatozoa and the Assessment of Their Post-Thawing Function," <i>Reprod. Fertil. Dev.</i> , 7:871-891 (1995).

EXAMINER 	DATE CONSIDERED 
--	---

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant(s).



UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
09/577,246	05/24/00	SCHENK	20091-201-01

HM22/1115
MCUTCHEEN DOYLE BROWN & PETERSEN LLP
THREE EMBARCADERO CENTER
SAN FRANCISCO CA 94111

EXAMINER

MELIER, M

ART UNIT	PAPER NUMBER
----------	--------------

1651

3

DATE MAILED:

11/15/00

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Office Action Summary

Application No.

09/577,246

Applicant(s)

Schenk

Examiner

Michael Meller

Group Art Unit

1651

☐ Responsive to communication(s) filed on _____

☐ This action is FINAL.

☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 35 C.D. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire one month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claim

- ☒ Claim(s) 27-37 is/are pending in the application.
- Of the above, claim(s) _____ is/are withdrawn from consideration.
- ☐ Claim(s) _____ is/are allowed.
- ☐ Claim(s) _____ is/are rejected.
- ☐ Claim(s) _____ is/are objected to.
- ☒ Claims 27-37 are subject to restriction or election requirement.

Application Papers

- ☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.
- ☐ The drawing(s) filed on _____ is/are objected to by the Examiner.
- ☐ The proposed drawing correction, filed on _____ is ☐ approved ☐ disapproved.
- ☐ The specification is objected to by the Examiner.
- ☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

- ☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).
- ☐ All ☐ Some* ☒ None of the CERTIFIED copies of the priority documents have been
- ☐ received.
- ☐ received in Application No. (Series Code/Serial Number) _____.
- ☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____

- ☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

- ☐ Notice of References Cited, PTO-892
- ☐ Information Disclosure Statement(s), PTO-1449, Paper No(s) _____
- ☐ Interview Summary, PTO-413
- ☐ Notice of Draftsperson's Patent Drawing Review, PTO-948
- ☐ Notice of Informal Patent Application, PTO-152

--- SEE OFFICE ACTION ON THE FOLLOWING PAGES ---

DETAILED ACTION

Election/Restriction

1. Restriction to one of the following inventions is required under 35 U.S.C. 121:
 - I. Claims 27-34, drawn to a product, classified in class 435, subclass 2.
 - II. Claim 35, drawn to a product by process, classified in class 435, subclass various.
 - III. Claims 36 and 37, drawn to a method of using the product, classified in class 424, subclass 520.
2. The inventions are distinct, each from the other because of the following reasons:

Inventions I and II are related as product and product-by-process. In the instant case the product can be made by a materially different process such as using a different final extender such as water or a salt.
3. Inventions I and III are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product (MPEP § 806.05(h)). In the instant case the product can be used as a substrate for an enzyme in an assay.

Art Unit: 1651

4. This application contains claims directed to the following patentably distinct species of the claimed invention: the species of claims 28-32, specifically what kind of sperm.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claims 27 and 29 are generic.

Applicant is advised that a reply to this requirement must include an identification of the species that is elected consonant with this requirement, and a listing of all claims readable thereon, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which are written in dependent form or otherwise include all the limitations of an allowed generic claim as provided by 37 CFR 1.141. If claims are added after the election, applicant must indicate which are readable upon the elected species. MPEP § 809.02(a).


Should applicant traverse on the ground that the species are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the species to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

Art Unit: 1651

5. Telephone calls were made to Emily Haliday on 11/6/200-11/8/2000 to request an oral election to the above restriction requirement, but did not result in an election being made since applicant's phone was busy every time the examiner called.

Applicant is advised that the reply to this requirement to be complete must include an election of the invention to be examined even though the requirement be traversed (37 CFR 1.143).

Any inquiries concerning this communication should be directed to Examiner Mike Meller at telephone number (703) 308-4230. If attempts to reach the examiner by telephone are unsuccessful, the Examiner's supervisor, Michael Wityshyn, can be reached at (703) 308-4743. The Fax phone number for the art unit is (703) 308- 0294. Any inquiries of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0196.


DAVID M. NAFF
PRIMARY EXAMINER
ART UNIT 1651

Docket No. 22091-701DIV2

#4
Req. Ext. (1 mo)
01/31/01

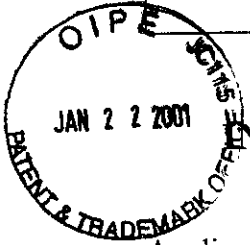
Certificate of Mailing/Transmission (37 C.F.R. § 1.8(a))

[X] Pursuant to 37 C.F.R. § 1.8, I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington D.C. 20231

Dated: January 16, 2001

Name of Person Certifying _____
Printed Name Nancy L. Hug

Nancy L. Hug



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE **RECEIVED**

Applicant: John Schenk
Filing Date: May 24, 2000
Serial No.: 09/577,246
Title: Method of Cryopreserving Selected Sperm Cells

Assignee: XY, Inc.
Examiner: M. Meller
Group Art Unit: 1651

JAN 26 2001

TECH CENTER 1600/2900

Assistant Commissioner for Patents
Washington, D.C. 20231

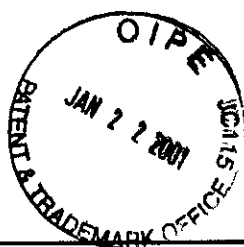
PETITION AND FEE FOR EXTENSION OF TIME
(37 C.F.R. § 1.136(a))

Sir:

Pursuant to 37 C.F.R. § 1.136(a), Applicant hereby petitions for a one month extension of time to respond to the Office Action mailed on November 15, 2000.

1. This communication is in connection with the matter for which this extension is requested
 - a. ☒ is filed herewith; or
 - b. ☐ has been filed on _____.

2. ☒ Applicant(s) claim Small Entity Status under 37 CFR § 1.27.



Docket No. 22091-701DIV2

3. The following fees are submitted:

	TOTAL MONTHS REQUESTED	OTHER THAN SMALL ENTITY	SMALL ENTITY	CALCULATIONS
a. <input checked="" type="checkbox"/>	one month	\$110.00	\$55.00	\$ 55.00
b. <input type="checkbox"/>	two month	\$390.00	\$195.00	\$
c. <input type="checkbox"/>	three month	\$890.00	\$445.00	\$
d. <input type="checkbox"/>	four month	\$1,390.00	\$695.00	\$
e. <input type="checkbox"/>	five month	\$1,890.00	\$945.00	\$
f. <input type="checkbox"/>	An extension for ____ months has already been secured for filing the above-identified communication and the fee paid therefor of \$ ____ is deducted from the total fee due for the total months of extension now requested. The fee for this extension (\$ ____), minus the fee previously paid (\$ ____) equals \$ ____ (total fee due).			\$
TOTAL FEES =				\$ 55.00

RECEIVED

JAN 26 2001

TECH CENTER 1600/2900

- ☒ Conditional Petition for Extension of Time: An extension of time is requested to provide for timely filing if an extension of time is still required after all papers filed with this communication have been considered.
- ☐ A check in the amount of \$ _____ to cover the above fees is enclosed.
- ☒ Please charge Deposit Account No. 50-1189, Docket No. 22091-701DIV2, in the amount of \$55.00 to cover the above-fees.
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-1189, Docket No. 22091-701DIV2.

DATE: January 16, 2001

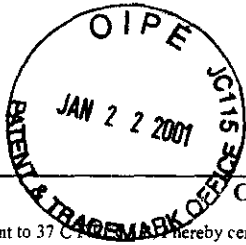
Respectfully submitted,

By:

Carol M. Gruppi

Registration No.: 37,341

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center
San Francisco, California 94111
Telephone: (415) 393-2000
Telefax: (415) 393-2286



Att 1651
✓

Docket No. 22091-701DIV2

Certificate of Mailing/Transmission (37 C.F.R. § 1.8(a)).
[X] Pursuant to 37 C.F.R. § 1.8(a), I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington D.C. 20231
Dated: January 16, 2001
Name of Person Certifying Nancy L. Hug
Printed Name: Nancy L. Hug

RECEIVED

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John Schenk
Filing Date: May 24, 2000
Serial No.: 09/577,246
Title: Method of Cryopreserving Selected Sperm Cells
Assignee: XY, Inc.
Examiner: M. Meller
Group Art Unit: 1651

JAN 26 2001
TECH CENTER 1600/2900

Assistant Commissioner for Patents
Washington, D.C. 20231

RESPONSE & FEE TRANSMITTAL

Sir:

In response to the Office Action mailed on November 15, 2000, enclosed herewith for filing are the following:

- ☐ A Response/Amendment [] page(s)
- ☒ A Response to Restriction Requirement under 35 USC § 121 [3] page(s)
- ☐ An Amendment Under 37 CFR § 1.111 [] page(s)
- ☐ An Amendment Under 37 CFR § 1.116 [] page(s)
- ☐ Other _____ [] page(s)

Also included are:

- ☒ A Petition for Extension of Time [1] month [2] page(s)
- ☐ Information Disclosure Statement
[] page(s) of PTO-1449 [] copies of IDS citations
- ☐ Applicant(s) claim Small Entity Status under 37 CFR § 1.27.
- ☐ Other: _____
- ☒ Return Postcard

Fee Calculation						
<input checked="" type="checkbox"/> The following fees are submitted:						CALCULATIONS
EXTRA CLAIMS FEE				OTHER THAN SMALL ENTITY	SMALL ENTITY	\$
CLAIMS	CURRENT #	# OF CLAIMS PREVIOUSLY PAID	# EXTRA	RATE	RATE	
Total Claims	37- 20	37	0	× \$18.00	× \$9.00	\$0
Independent claims	1- 3	1	0	× \$80.00	× \$40.00	\$0
MULTIPLE DEPENDENT CLAIM(S)						
<input type="checkbox"/> Yes <input type="checkbox"/> No				\$270.00	\$135.00	\$
Petition for Extension of Time Fee (1 months)						\$ 55.00
OTHER FEES _____ (specify)						\$
TOTAL FEES =						\$55.00

- ☒ Conditional Petition for Extension of Time: An extension of time is requested to provide for timely filing if an extension of time is still required after all papers filed with this communication have been considered.
- ☐ A check in the amount of \$ _____ to cover the above fees is enclosed.
- ☒ Please charge Deposit Account No. 50-1189, Docket No. 22091-701DIV2, in the amount of \$55.00 to cover the above-fees.
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-1189, Docket No. 22091-701DIV2.

DATE: January 16, 2001

Respectfully submitted,

By: _____

Carol M. Gruppi

Registration No.: 37,341

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center
San Francisco, California 94111
Telephone: (415) 393-2000
Telefax: (415) 393-2286

<p align="center">Certificate of Mailing/Transmission (37 C.F.R. § 1.8(a))</p> <p>I hereby certify that this correspondence is, on the date shown below, being:</p>	
<p align="center">MAILING</p> <p><input checked="" type="checkbox"/> deposited with the United States Postal Service as First Class Mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington, D C 20231</p>	<p align="center">FACSIMILE</p> <p><input type="checkbox"/> transmitted by facsimile to the Patent and Trademark Office</p>
<p>Date <u>January 16, 2001</u></p>	<p>Name of Person Certifying <u>Carol M. Grupp</u></p>

#5
Elee
1/31/01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s): John Schenk

Assignee: XY, Inc.

Serial No.: 09/577,246

Examiner: M. Meller

Filing Date: May 24, 2000

Group Art Unit: 1651

Title: Method of Cryopreserving Selected Sperm Cells



ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

RESPONSE TO RESTRICTION REQUIREMENT UNDER 35 U.S.C. § 121

Dear Sir:

This is in response to the Office Action dated November 15, 2000. This response is accompanied by a petition for a one month extension of time and the appropriate fee under 37 C.F.R. § 1.16 and § 1.17. Accordingly this response is timely filed.

The Office Action dated November 15, 2000, stated that there were distinct inventions and required Applicants to elect one invention for prosecution on the merits. The distinct inventions, as set forth in the Restriction Requirement, are as follows:

- I. Claims 27-34, drawn to a product , classified in class 435, subclass 2;
- II. Claim 35, drawn to a product by process, classified in class 435, subclass various;
and
- III. Claims 36 and 37, drawn to a method of the using the product, classified in class 424, subclass 520.

Applicants hereby provisionally elect the Claims of Group 1 for prosecution on the merits.

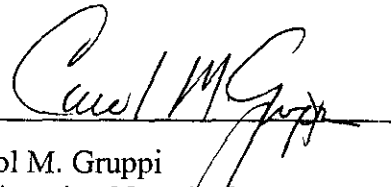
The Restriction Requirement also stated that the Claims were directed to patentably distinct species. Specifically, election of a species for “what kind of sperm” recited in Claim 28-32 for prosecution on the merits was requested. The Examiner stated that Claims 27 and 29 are generic. As a species for what kind of sperm, Applicant provisionally selected sperm and bovine (as the type of mammalian sperm) for the type of sperm. The claims readable on these species are 27-34.

Applicants, however, traverse the Restriction Requirement on the basis that it would not be unduly burdensome for the Examiner to search more than one set of claims or patentably distinct species. A Restriction Requirement requires the inventions be independent or distinct as claimed, and there must be a serious burden on the Examiner for restriction to be required (*see* MPEP § 803). Ten independent and distinct nucleotide sequences have been determined to be a “reasonable number” of sequences to be claimed and examined in a single application without restriction (*see* MPEP § 803.04). Thus, examination of ten independent and distinct nucleotide sequences has been determined not constitute an undue burden for the Examiner (*see* MPEP § 803.04). Likewise, Applicants submit that it would not be unduly burdensome for the Examiner to search for more than one additional type of sperm. Accordingly, reconsideration of the election of species is respectfully requested. If Examiner has any questions concerning this Response, the Examiner is respectfully requested to telephone Applicant’s agent at the telephone number given below.

Docket No: 22091-701DIV2

A fee of \$55.00 is due under 37 C.F.R. § 1.16 and § 1.17 for the Petition for a an Extension of Time filed concurrently herewith. The Assistant Commissioner is hereby authorized to charge any additional fees which may be required by this paper, or credit any overpayment to Deposit Account No. 50-1189. Docket No.: 22091-701DIV2.

Respectfully submitted,

By: 
Carol M. Gruppi
Registration No.: 37,341

Dated: January 16, 2000

Mailing Address:

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center
San Francisco, California 94111
Telephone: (650) 849-4400
Telefax: (650) 849-4800



**UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office**

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
-----------------	-------------	----------------------	---------------------

07/5/77, 246 05/24/00 SCHENF

J 22091-701 DJ

EXAMINER

HM12/0228

MCCUTCHEN DOYLE BROWN & ENERSEN LLP
THREE EMBARCADERO CENTER
SAN FRANCISCO CA 94111

MELLER, M

ART UNIT

PAPER NUMBER

1651

DATE MAILED:

02/28/01

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Office Action Summary	Application No. 09/577,246		Applicant(s) SCHENK, JOHN L.	
	Examiner Michael V. Meller		Art Unit 1651	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136 (a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) ☒ Responsive to communication(s) filed on 22 January 2001.

2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.

3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4) ☒ Claim(s) 27-37 is/are pending in the application.

4a) Of the above claim(s) 35-37 is/are withdrawn from consideration.

5) ☐ Claim(s) _____ is/are allowed.

6) ☒ Claim(s) 27-34 is/are rejected.

7) ☐ Claim(s) _____ is/are objected to.

8) ☐ Claims _____ are subject to restriction and/or election requirement.

Application Papers

9) ☐ The specification is objected to by the Examiner.

10) ☐ The drawing(s) filed on _____ is/are objected to by the Examiner.

11) ☐ The proposed drawing correction filed on _____ is: a) ☐ approved b) ☐ disapproved.

12) ☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

13) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a) ☐ All b) ☐ Some * c) ☐ None of:

1. ☐ Certified copies of the priority documents have been received.

2. ☐ Certified copies of the priority documents have been received in Application No. _____.

3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14) ☒ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

15) <input checked="" type="checkbox"/> Notice of References Cited (PTO-892) 16) <input type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948) 17) <input checked="" type="checkbox"/> Information Disclosure Statement(s) (PTO-1449) Paper No(s) <u>1</u> .	18) <input type="checkbox"/> Interview Summary (PTO-413) Paper No(s). _____ 19) <input type="checkbox"/> Notice of Informal Patent Application (PTO-152) 20) <input type="checkbox"/> Other: _____
--	--

DETAILED ACTION

Election/Restrictions

Applicant's election with traverse of Group I, claims 27-34 and sex selected bovine sperm in Paper No. 5 is acknowledged. The traversal is on the ground(s) that it would not be unduly burdensome for the examiner to search more than one set of claims or patentably distinct species. This is not found persuasive because as the applicant is aware, the claims require an extensive literature search in the area of biotechnology which is not co-extensive. The examiner has provided specific reasons in the previous office action which applicant has not rebutted, and applicant is reminded that this case does not involve sequences, i.e. nucleic or amino acid.

Claims 35-37 are withdrawn from further consideration by the examiner.

The requirement is still deemed proper and is therefore made FINAL.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 27-34 are rejected under 35 U.S.C. 103(a) as being unpatentable over any one of Seidel et al. '689 (see entire reference, especially col. 1, first paragraph, examples 1 and 2), Seidel et al. '867 (see entire reference, especially col. 1, first paragraph, examples 1 and 2), Chandler (see example 7), Johnson (see abstract, col. 3, lines 18-21, example 1) or Spaulding (see abstract, examples 1, 2 and 10) taken with any one of Salisbury et al. (see pages 494-539), Critser et al. (see entire reference especially, col. 1, lines 14-26) , or Behrman et al. (see entire reference, especially, Tables II, IV).

Each of the primary references teaches a sex-selected bovine sperm wherein the sperm has been selected by flow cytometry. They do not teach a sperm which is frozen after such a process.

It would have been obvious to one of ordinary skill in the art to freeze the sperm of the primary references after the sex selection (Seidel et al. '689 ,Seidel et al. '867 , Chandler , Johnson and Spaulding) since the secondary references (Salisbury et al. , Critser et al. and Behrman et al.) each teach freezing of sperm and the benefits of doing such, i.e. self-life, stability, storage stability, etc. and since one would want to preserve the sperm if he/she was not going to use it right away.

Application/Control Number: 09/577,246
Art Unit: 1651


Page 4

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michael V. Meller whose telephone number is 703-308-4230. The examiner can normally be reached on Monday thru Friday: 10:30am-7:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Michael Wityshyn can be reached on 703-308-4743. The fax phone numbers for the organization where this application or proceeding is assigned are 703-308-0294 for regular communications and 703-308-0294 for After Final communications.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is 703-308-0196.

MVM
February 26, 2001


DAVID M. NAFF
PRIMARY EXAMINER
ART UNIT 1651

Notice of References Cited	Application/Control No. 09/577,246	Applicant(s)/Patent Under Reexamination SCHENK, JOHN L	
	Examiner Michael V Meller	Art Unit 1651	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification	
	A	US-6149867-	11-2000	Seidel et al.	422	73
	B	US-6071689-	06-2000	Seidel et al.	435	2
	C	US-5691133-	11-1997	Critser et al.	435	2
	D	US-5021244-	06-1991	Spaulding	424	561
	E	US- -				
	F	US- -				
	G	US- -				
	H	US- -				
	I	US- -				
	J	US- -				
	K	US- -				
	L	US- -				
	M	US- -				

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification	
	N	- -					
	O	- -					
	P	- -					
	Q	- -					
	R	- -					
	S	- -					
	T	- -					

NON-PATENT DOCUMENTS

*		Include as applicable. Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action (See MPEP § 707.05(a))
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign



RECEIVED

JUN 22 2001

TECH CENTER 1600/2900

Docket No. 22091-701DIV2

Certificate of Mailing/Transmission (37 C.F.R. § 1.8(a))

[X] Pursuant to 37 C.F.R. § 1.8, I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington D.C. 20231

[] Pursuant to 37 C.F.R. § 1.6(d), I hereby certify that this paper and all enclosures are being sent via facsimile on the date indicated below to the attention of Examiner _____ at Facsimile No. _____ at _____ a.m./p.m.

Dated: June 13, 2001

Name of Person Certifying: Nancy L. Hug
Printed Name: Nancy L. Hug

Handwritten: # 11008 6/25/01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	John L. Schenk	Assignee:	XY, Inc.
Filing Date:	05/24/00	Examiner:	M. Meller
Serial No.:	09/577,246	Group Art Unit:	1651
Title:	METHOD OF CRYOPRESERVING SELECTED SPERM CELLS		

Assistant Commissioner for Patents
Washington, D.C. 20231

INFORMATION DISCLOSURE STATEMENT

Sir:

In accordance with 37 C.F.R. § 1.56, the references listed on the attached Form PTO-1449 are being brought to the attention of the Examiner for consideration in connection with the examination of the above-identified patent application.

I. Timing of the Information Disclosure Statement:

This Information Disclosure Statement is filed:

- ☐ With the new patent application submitted herewith (37 C.F.R. § 1.97(a)).
- ☐ Within three months after the filing date of the application or within three months after the date of entry of the national stage of a PCT application as set forth in 37 C.F.R. § 1.491.
- ☐ Before the mailing date of a first Office action on the merits. In the event, however, that an Office Action has crossed in the mail with this Information Disclosure Statement, the Commissioner is hereby authorized to charge Deposit Account No. [] for any fees required pursuant to 37 C.F.R. §§ 1.17(p) or 1.17(i)(1).

This Information Disclosure Statement is filed:

- ☐ After the first Office Action and more than three months after the application's filing date; or PCT national stage date of entry filing but, as far as is known to the undersigned, prior to the mailing date of either a final rejection or a notice of allowance, whichever occurs first, and the Commissioner is hereby authorized to charge Deposit Account No. [] for the fee (\$180) set forth in 37 C.F.R. § 1.17(p) and any additional required fees.

This Information Disclosure Statement is filed:

- ☐ After the mailing date of either a final rejection or a notice of allowance, whichever occurred first, and is accompanied by the fee (\$180.00) set forth in 37 C.F.R. § 1.17(i)(1) and a certification as specified in 37 C.F.R. § 1.97(e), as checked below. This document is to be considered as a petition requesting consideration of the Information Disclosure Statement.

The undersigned certifies that:

- ☒ Each item of information contained in the Information Disclosure Statement was the first citation of that item by a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this information disclosure statement.
- ☐ No item of information contained in this information disclosure statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application or, to the knowledge of the undersigned after making reasonable inquiry, was known to any individual designated in 37 C.F.R. § 1.56(c) more than three months prior to the filing of this Information Disclosure Statement.

II. Copies of the Cited Items:

- ☒ Copies of all of the items listed on the attached Form PTO-1449 are enclosed.
- ☐ Copies of only the following items listed on the attached Form PTO-1449 are enclosed: _____.
- ☐ Copies of those items which are marked with an asterisk (*) in the attached Form PTO-1499 are not supplied because they were previously cited by or submitted to the Patent Office in a prior Application No. _____, filed _____ and relied upon in this application for an earlier filing date under 35 U.S.C § 120. See 37 C.F.R. § 1.98(d).

- ☒ A copy of the search report and the cited references not already of record in this application are attached hereto.

III. Concise Explanation of Relevance:

- ☒ A concise explanation of relevance of the items listed on Form PTO-1449 is not given.
- ☐ A concise explanation of relevance of [some of] the items listed on Form PTO-1449 is in the form of an English language copy of a Search Report from a foreign patent office, issued in a counterpart application, which refers to the relevant portions of the references (copy attached).

IV. Conclusion:

Citation of the above documents shall not be construed as:

1. an admission that the documents are necessarily prior art with respect to the instant invention;
2. a representation that a search has been made, other than as described above; or
3. an admission that the information cited herein is, or is considered to be, material to patentability as defined in § 1.56(b).

It is respectfully requested that the Examiner indicate consideration of the cited references by returning a copy of the attached form PTO 1449 with initials or other appropriate marks.

The Commissioner is hereby authorized to charge Deposit Account No. 50-1189 Docket No.: 22091-701 for any additional fees required in connection with the filing of this Information Disclosure Statement.

DATE: June 13, 2001

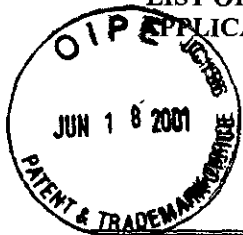
Respectfully submitted,

By: _____

Carol M. Gruppi

Registration No.: 37,841

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center
San Francisco, California 94111
Telephone: (415) 393-2000
Telefax: (415) 393-2286



FORM PTO-1449 (Modified [6-1])

LIST OF PATENTS AND PUBLICATIONS FOR
APPLICANT(S)' INFORMATION DISCLOSURE
STATEMENT

(Use several sheets if necessary)

ATTY. DOCKET NO.

22091-0701DIV2

SERIAL NO.

09/577,246

INVENTOR

John L. Schenk

FILING DATE

05/24/00

GROUP ART UNIT

1651

REFERENCE DESIGNATION

U.S. PATENT DOCUMENTS

EXAM'R INITIAL		DOCUMENT NUMBER	DATE	NAME	Class	Subclass	Filing Date If Appropriate
MM	A1	4,474,875	10/2/84	Shrimpton Wallace	435	2	
	A2						
	A3						

FOREIGN PATENT DOCUMENTS

EXAM'R INITIAL		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	Subclass	TRANSLAT'N
							yes no
	B1						
	B2						
	B3						
	B4						
	B5						

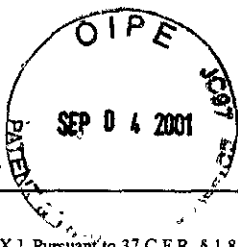
OTHER ART (Include Author, Title, Date, Pertinent Pages, etc.)

MM	C1	Database WPI, Section CH, Week 199920; Derwent Publications Ltd., London, GB; Class B04, AN 1999-229816; XP002164167 & BR 9704313 (Alves EW, De Resende Matta M F et al (April 6, 1999 - Abstract).
	C2	DeLeeuw, F E et al. "Effects Of Various Cryoprotective Agents And Membrane-Stabilizing Compounds On Bull Sperm Membrane Integrity After Cooling And Freezing," (1993), <i>Cryobiology</i> , US, Academic Press Inc.; Vol. 30, No. 1, pp. 32-44, XP002089949; ISSN: 0011-2240.
	C3	Stap, J. et al, "Improving The Resolution Of Cryopreserved X- and Y-Sperm During DNA Flow Cytometric Analysis" (1998), <i>J Anim. Sci.</i> ; Vol. 76, pp. 1896-1902, XP000985715
	C4	

EXAMINER

DATE CONSIDERED

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant(s).



Docket No. 22091-701DIV2

Certificate of Mailing (37 C.F.R. § 1.8(a)):

[X] Pursuant to 37 C.F.R. § 1.8, I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington D.C. 20231

Dated: August 28, 2001

Name of Person Certifying: Carol M. Gruppi

Printed Name: Carol M. Gruppi

#8
890
9/8/01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John Schenk

Assignee: XY, Inc.

Serial No.: 09/577,246

Examiner: M. Meller

Filing Date: May 24, 2000

Group Art Unit: 1651

Title: Method of Cryopreserving Selected Sperm Cells

Commissioner for Patents
Washington, D.C. 20231

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SEP 06 2001
TECH CENTER 1600/2900

PETITION AND FEE FOR EXTENSION OF TIME
(37 C.F.R. § 1.136(a))

Sir:

Pursuant to 37 C.F.R. § 1.136(a), Applicant hereby petitions for a three month extension of time to respond to the Office Action mailed on February 28, 2001.

1. This communication is in connection with the matter for which this extension is requested

- a. ☒ is filed herewith; or
b. ☐ has been filed on _____.

2. ☒ Applicant(s) claim Small Entity Status under 37 CFR § 1.27.

3. The following fees are submitted:

	TOTAL MONTHS REQUESTED	OTHER THAN SMALL ENTITY	SMALL ENTITY	CALCULATIONS
a. <input type="checkbox"/>	one month	\$110.00	\$55.00	\$
b. <input type="checkbox"/>	two month	\$390.00	\$195.00	\$
c. <input checked="" type="checkbox"/>	three month	\$890.00	\$445.00	\$445.00
d. <input type="checkbox"/>	four month	\$1,390.00	\$695.00	\$
e. <input type="checkbox"/>	five month	\$1,890.00	\$945.00	\$
f. <input type="checkbox"/>	An extension for ____ months has already been secured for filing the above-identified communication and the fee paid therefor of \$ ____ is deducted from the total fee due for the total months of extension now requested. The fee for this extension (\$ ____), minus the fee previously paid (\$ ____) equals \$ ____ (total fee due).			\$
TOTAL FEES =				\$445.00

- ☒ Conditional Petition for Extension of Time: An extension of time is requested to provide for timely filing if an extension of time is still required after all papers filed with this communication have been considered.
- ☐ A check in the amount of \$ _____ to cover the above fees is enclosed.
- ☒ Please charge Deposit Account No. 50-1189, Docket No. 22091-701DIV2, in the amount of \$445.00 to cover the above-fees.
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-1189, Docket No. 22091-701DIV2..

DATE: August 28, 2001

Respectfully submitted,

By: _____

Carol M. Gruppi

Registration No.: 37,341

McCutchen, Doyle, Brown & Enersen, LLP
 Three Embarcadero Center, Suite 1800
 San Francisco, California 94111
 Telephone: (415) 393-2000
 Telefax: (415) 393-2286



Docket No. 22091-701DIV2

Certificate of Mailing (37 C.F.R. § 1.8(a))	
[X] Pursuant to 37 C.F.R. § 1.8, I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington D.C. 20231.	
Dated: <u>August 28, 2001</u>	Name of Person Certifying: <u><i>Carol M. Gruppi</i></u> Printed Name: Carol M. Gruppi

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	John Schenk	Assignee:	XY, Inc.
Serial No.:	09/577,246	Examiner:	M. Meller
Filing Date:	May 24, 2000	Group Art Unit:	1651
Title:	Method of Cryopreserving Selected Sperm Cells		

Commissioner for Patents
Washington, D.C. 20231

PETITION AND FEE FOR EXTENSION OF TIME
(37 C.F.R. § 1.136(a))

RECEIVED
SEP 06 2001
TECH CENTER 1600/2900

Sir:

Pursuant to 37 C.F.R. § 1.136(a), Applicant hereby petitions for a three month extension of time to respond to the Office Action mailed on February 28, 2001.

- 1. This communication is in connection with the matter for which this extension is requested
 - a. ☒ is filed herewith; or
 - b. ☐ has been filed on _____.
- 2. ☒ Applicant(s) claim Small Entity Status under 37 CFR § 1.27.

3. The following fees are submitted:

	TOTAL MONTHS REQUESTED	OTHER THAN SMALL ENTITY	SMALL ENTITY	CALCULATIONS
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b. <input type="checkbox"/>	two month	\$390.00	\$195.00	\$
c. <input checked="" type="checkbox"/>	three month	\$890.00	\$445.00	\$445.00
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e. <input type="checkbox"/>	five month	\$1,890.00	\$945.00	\$
f. <input type="checkbox"/>	An extension for ____ months has already been secured for filing the above-identified communication and the fee paid therefor of \$ ____ is deducted from the total fee due for the total months of extension now requested. The fee for this extension (\$ ____), minus the fee previously paid (\$ ____) equals \$ ____ (total fee due).			\$
TOTAL FEES =				\$445.00

- ☒ Conditional Petition for Extension of Time: An extension of time is requested to provide for timely filing if an extension of time is still required after all papers filed with this communication have been considered.
- ☐ A check in the amount of \$ _____ to cover the above fees is enclosed.
- ☒ Please charge Deposit Account No. 50-1189, Docket No. 22091-701DIV2, in the amount of \$445.00 to cover the above-fees.
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-1189, Docket No. 22091-701DIV2..

DATE: August 28, 2001

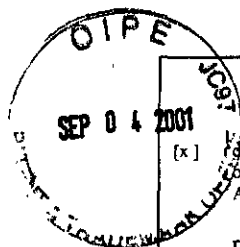
Respectfully submitted,

By: _____

Carol M. Gruppi

Registration No.: 37,341

McCutchen, Doyle, Brown & Enersen, LLP
 Three Embarcadero Center, Suite 1800
 San Francisco, California 94111
 Telephone: (415) 393-2000
 Telefax: (415) 393-2286



Certificate of Mailing/Transmission (37 C.F.R. § 1.8(a))	
I hereby certify that this correspondence is, on the date shown below, being:	
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<input checked="" type="checkbox"/> deposited with the United States Postal Service as First Class Mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.	<input type="checkbox"/> transmitted by facsimile to the Patent and Trademark Office
Date: <u>August 28, 2001</u>	Name of Person Certifying: <u>Carol M. Grupp</u>

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s): John Schenk

Assignee: XY, Inc.

Serial No.: 09/577,246

Examiner: M. Meller

Filing Date: May 24, 2000

Group Art Unit: 1651

Title: Method of Cryopreserving Selected Sperm Cells

TECH CENTER 1600/2900

SEP 06 2001

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COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

AMENDMENT UNDER 37 C.F.R. § 1.111

Sir:

This is in response to the Office Action dated February 28, 2001. This response is accompanied by a petition for an extension of time to extend the period to respond to the Office Action to August 28, 2001, and the appropriate fee under 37 C.F.R. § 1.16 and § 1.17. Please amend the application as follows.

CLAIMS

Please add the following new claims:

38. (New) The frozen selected sperm sample of Claim 27, wherein the concentration of sperm prior to freezing was about $1 \times 10^6/\text{ml}$ to about $300 \times 10^6/\text{ml}$.
39. (New) The frozen selected sperm sample of Claim 27, wherein the concentration of sperm prior to freezing was about $10 \times 10^6/\text{ml}$ to about $50 \times 10^6/\text{ml}$.

40. (New) The frozen selected sperm sample of Claim 27, wherein the concentration of sperm prior to freezing was about $10 \times 10^6/\text{ml}$ to about $20 \times 10^6/\text{ml}$.

41. (New) A frozen selected sperm sample comprising sperm selected for a characteristic, wherein the method used to select said selected sperm is flow cytometry.

42. (New) The frozen selected sperm sample of Claim 41, wherein the method used to select said selected sperm is fluorescent activated cell sorting.

43. (New) The frozen selected sperm sample of Claim 41, wherein the concentration of sperm prior to freezing was about $1 \times 10^6/\text{ml}$ to about $300 \times 10^6/\text{ml}$.

44. (New) The frozen selected sperm sample of Claim 41, wherein the concentration of sperm prior to freezing was about $10 \times 10^6/\text{ml}$ to about $50 \times 10^6/\text{ml}$.

45. (New) The frozen selected sperm sample of Claim 41, wherein the concentration of sperm prior to freezing was about $10 \times 10^6/\text{ml}$ to about $20 \times 10^6/\text{ml}$.

46. (New) The frozen selected sperm sample of Claim 41, wherein said selected sperm is sex selected sperm.

47. (New) The frozen selected sperm sample of Claim 41 wherein said frozen selected sperm sample comprises mammalian sperm.

48. (New) The frozen selected sperm sample of Claim 47 wherein said frozen selected sperm sample comprises bovine sperm.

49. (New) The frozen selected sperm sample of Claim 47, wherein said frozen selected sperm sample comprises equine sperm.

50. (New) The frozen selected sperm sample of Claim 47 wherein said frozen selected sperm sample comprises porcine sperm.

REMARKS

Claims 27 -37 are pending in this application. Claims 27 -34 stand rejected. Claims 35-37 have been withdrawn from consideration. New claims 38-50 have been added. Support for the newly added claims may be found throughout the specification. For example, support for new claims 38-39 may be found at page 11, lines 24-28; for claims 41 and 42 at page 6, lines 7-19; claims 43-45 at page 11, lines 24-28; claim 46 at page 6, lines 7-19; claims 47-50 at pages 2 and 3, bridging paragraph. Thus, no new matter has been added by these amendments. A copy

of the currently pending claims (including the newly added claims) is attached hereto as Exhibit A.

Reconsideration of the application in view of the following remarks is respectfully requested.

REJECTIONS UNDER 35 U.S.C. § 103(a)

Claims 27-33 also stand rejected under 35 U.S.C. § 103(a) as being unpatentable over any one of Seidel et al (U.S. Patent No. 6,071,689), Seidel et al (U.S. Patent No. 6,149,867), Chandler (U.S. Patent No. 5,514,537), Johnson (U.S. Patent No. 5,135,759) or Spaulding (U.S. Patent No. 5,012,244) taken with any one of Salisbury et al (*Physiology of Reproduction and Artificial Insemination of Cattle* (1978), 2nd Edition, pages 494-539), Crister (U.S. Patent No. 5,691,133), or Behrman et al (*Am J. Obst. & Gynec* March 1, 1969). Applicant respectfully traverses this rejection for the reasons set forth below.

The instantly claimed invention is directed to a *frozen selected sperm sample* comprising a portion of the sperm present in a source sample which has been selected for a certain characteristic. Thawing of the frozen selected sperm sample results in *viable* (e.g., produces live offspring), *selected* (e.g., sex selected) *sperm* that can be used in procedures such as artificial insemination ("AI") and in vitro fertilization ("IVF"). Frozen selected sperm samples which, when thawed, result in viable selected sperm, greatly facilitates the storage and/or shipment of selected sperm samples and allows widespread use of selected sperm. The ease of shipment and the resulting availability of the frozen selected sperm samples, enables an increase in production efficiency and production quality of offspring (e.g., livestock, show or race horses, endangered species) having desirable traits. Thus, the instant invention represents an important advance in animal husbandry.

Prior researchers had demonstrated that the stresses associated with various selection methods or with cryopreservation resulted in significant losses in fertility and/or viability of sperm. The selection and subsequent processing of sperm presents unique challenges because sperm are relatively susceptible to physical injury, are incapable of DNA repair and exhibit decreased fertility with increasing time between collection and use. As most of the available selection methods involve physical stresses and take time, selected sperm are compromised compared to non-selected sperm. Fertility is generally further reduced if the selection technique (e.g., flow cytometry) involves significant dilution (see specification, page 1, lines 28-31, page 2,

lines 1-5). In addition, the freezing of sperm invariably reduces fertility, motility, and/or viability (see specification, page 2, lines 15-17). Although techniques for freezing unselected sperm are known, *no technique for repeatedly producing viable cryopreserved selected sperm has been described prior to the instant disclosure.*

Moreover, prior to applicant's invention, no viable pregnancies had been achieved or no offspring exhibiting the selected characteristic had been produced using selected cryopreserved sperm. Thus, for one of ordinary skill in the art, there would be no reasonable expectation of success in using cryopreserved selected sperm to produce pregnancies which resulted in offspring with the selected characteristic. The present inventors have demonstrated, for the first time, that pregnancies can be achieved with sperm that have been selected and then frozen and that the resulting offspring exhibit the selected characteristic. (See, e.g. Example 8). Thus, Applicant's invention meets a long felt need in the field of production agriculture and animal husbandry (e.g., dairy cattle, horses, endangered species etc).

Seidel et al '689 and Seidel et al '867 relate to improved sheath and collector systems for sex selecting sperm via flow cytometry. Chandler '537 relates to a method and apparatus for sex sperm based on the size differential between the X and Y chromosome. Johnson '759 relates to a method for sex selecting sperm based on DNA content utilizing flow cytometry or cell sorter. Neither Seidel ('689 or '867), Chandler ('537) or Johnson (759) teach or suggest that sorted sperm may be frozen. Thus, neither Seidel, Chandler or Johnson can render the claimed invention obvious either alone or in combination.

Spaulding '244 relates to a method for sex selecting sperm utilizing sex associated membrane antibodies. In Spaulding's method, an antibody targeting either the X or Y membrane is added to a sperm sample. The sperm targeted by the antibody is not "sorted" from the sample, (e.g., instant claims 41-50), but is inactivated by the antibody and remains in the sample with the inactivated antibody. The entire sperm sample (i.e., both activated and inactivated sperm), not just a portion comprising selected sperm, is used in the *in vitro* fertilization. In addition, Spaulding does not teach or suggest that sperm selected for a particular characteristic, frozen, and thawed will be viable (i.e., that pregnancies can be achieved with such selected cryopreserved sperm.). Accordingly, Spaulding, either alone or in combination, does not render the claimed invention obvious.

Salisbury is a general review relating to artificial insemination in cattle. Salisbury discusses techniques *for freezing unselected sperm*. Behrman et al relates to the freezing of unselected human sperm. As noted by the Examiner, neither Salisbury or Behrman teach or suggest freezing selected sperm. Salisbury or Behrman do not remedy the deficiencies of the primary references. Such a disclosure is found only in the instant application. Accordingly, neither Salisbury or Behrman, either alone or in combination render the claimed invention obvious.

Applicant respectfully submits that a proper analysis under 35 USC § 103 requires a consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process; and (2) whether the prior art would also have revealed that in so making or carrying out those of ordinary skill would have a reasonable expectation of success. Both the suggestion and the reasonable expectation of success must be found in the prior art, not applicant's disclosure. In re Vaeck 947 Fed, 2d 488, 493, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991). The cryopreservation of selected sperm (e.g., sperm selected by flow cytometry) and/or the use of such sperm to achieve viable pregnancies which result in offspring with the selected characteristic is found only in the instant application, and not in the cited references. Absent such teachings, the cited references cannot render the claimed invention obvious. Accordingly, applicant respectfully requests withdrawal of this ground of rejection.

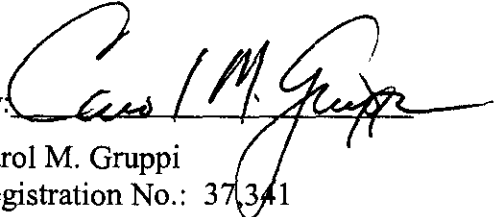
CONCLUSION

Applicants respectfully submit that the claims define an invention that is patentable over the art. Accordingly, allowance is in order, and an early notification to that effect would be appreciated. Should the Examiner in reviewing the communication have any questions or need any additional information, he is welcome to contact the undersigned at (650) 849-4902.

A fee of \$481.00 is due under 37 C.F.R. § 1.16 and § 1.17 for the Petition for a an Extension of Time and additional claims filed concurrently herewith. The Assistant Commissioner is hereby authorized to charge any additional fees which may be required by this paper, or credit any overpayment to Deposit Account No. 50-1189. Docket No.: 22091-701DIV2.

Respectfully submitted,

By:


Carol M. Gruppi
Registration No.: 37,341

Dated: August 28, 2001

Mailing Address:

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center, Suite 1800
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Telephone: (650) 849-4902
Telefax: (650) 849-4800
e-mail: cgruppi@mdbe.com

A

Claims Pending in U.S.S.N.: 09/577,246

27. A frozen selected sperm sample comprising a portion of the sperm present in a source sample, said portion of sperm selected for a characteristic.
28. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample comprises sex-selected sperm.
29. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample comprises mammalian sperm.
30. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample comprises bovine sperm.
31. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample comprises equine sperm.
32. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample comprises porcine sperm.
33. The frozen selected sperm sample of Claim 27 wherein the method used to select said selected sperm sample comprises a technique from the group consisting of flow cytometry, a magnetic technique, a columnar technique, a gravimetric technique, a biochemical technique, a technique based on motility of sperm, a technique based on an electrical property of sperm, and any combination thereof.
34. The frozen selected sperm sample of Claim 33 wherein said frozen selected sperm sample comprises sperm that have been selected by flow cytometry.
38. (New) The frozen selected sperm sample of Claim 27, wherein the concentration of sperm prior to freezing was about $1 \times 10^6/\text{ml}$ to about $300 \times 10^6/\text{ml}$.

39. (New) The frozen selected sperm sample of Claim 27, wherein the concentration of sperm prior to freezing was about $10 \times 10^6/\text{ml}$ to about $50 \times 10^6/\text{ml}$.
40. (New) The frozen selected sperm sample of Claim 27, wherein the concentration of sperm prior to freezing was about $10 \times 10^6/\text{ml}$ to about $20 \times 10^6/\text{ml}$.
41. (New) A frozen selected sperm sample comprising sperm selected for a characteristic, wherein the method used to select said selected sperm is flow cytometry.
42. (New) The frozen selected sperm sample of Claim 41, wherein the method used to select said selected sperm is fluorescent activated cell sorting.
43. (New) The frozen selected sperm sample of Claim 41, wherein the concentration of sperm prior to freezing was about $1 \times 10^6/\text{ml}$ to about $300 \times 10^6/\text{ml}$.
44. (New) The frozen selected sperm sample of Claim 41, wherein the concentration of sperm prior to freezing was about $10 \times 10^6/\text{ml}$ to about $50 \times 10^6/\text{ml}$.
45. (New) The frozen selected sperm sample of Claim 41, wherein the concentration of sperm prior to freezing was about $10 \times 10^6/\text{ml}$ to about $20 \times 10^6/\text{ml}$.
46. (New) The frozen selected sperm sample of Claim 41, wherein said selected sperm is sex selected sperm.
47. (New) The frozen selected sperm sample of Claim 41 wherein said frozen selected sperm sample comprises mammalian sperm.
48. (New) The frozen selected sperm sample of Claim 47 wherein said frozen selected sperm sample comprises bovine sperm.
49. (New) The frozen selected sperm sample of Claim 47, wherein said frozen selected sperm sample comprises equine sperm.

Claims Pending in U.S.S.N.: 09/577,246 (continued)

50. (New) The frozen selected sperm sample of Claim 47 wherein said frozen selected sperm sample comprises porcine sperm.



JIP-1651\$

Docket No. 22091-701DIV2

Certificate of Mailing (37 C.F.R. § 1.8(a))	
[X] Pursuant to 37 C.F.R. § 1.8, I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Washington D.C. 20231.	
Dated <u>August 28, 2001</u>	Name of Person Certifying: <u>Carol M. Gruppi</u> Printed Name: Carol M. Gruppi

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s):	John Schenk	Assignee:	XY, Inc.
Serial No.:	09/577,246	Examiner:	M. Meller
Filing Date:	May 24, 2000	Group Art Unit:	1651
Title:	Method of Cryopreserving Selected Sperm Cells		

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Commissioner for Patents
Washington, D.C. 20231

AMENDMENT & FEE TRANSMITTAL

Sir:

In response to the Office Action mailed on February 28, 2001, enclosed herewith for filing are the following:

- ☒ An Amendment [6] pages plus Exhibit A [3] pages
- ☐ A Response to Restriction Requirement under 35 USC § 121 [] page(s)
- ☐ An Amendment Under 37 CFR § 1.111 [] page(s)
- ☐ An Amendment Under 37 CFR § 1.116 [] page(s)
- ☐ Other _____ [] page(s)

Also included are:

- ☒ A Petition for Extension of Time [3] months [2] page(s)
- ☐ Information Disclosure Statement
[] page(s) of PTO-1449 [] copies of IDS citations
- ☒ Applicant(s) claim Small Entity Status under 37 CFR § 1.27.
- ☐ Other: _____
- ☒ Return Postcard

Fee Calculation						
<input checked="" type="checkbox"/> The following fees are submitted:						CALCULATIONS
EXTRA CLAIMS FEE				OTHER THAN SMALL ENTITY	SMALL ENTITY	
CLAIMS	CURRENT #	# OF CLAIMS PREVIOUSLY PAID	# EXTRA	RATE	RATE	
Total Claims	20-	24	4	× \$18.00	× \$9.00	\$36.000
Independent claims	3 -	2	0	× \$80.00	× \$40.00	\$0
MULTIPLE DEPENDENT CLAIM(S)						
<input type="checkbox"/> Yes <input type="checkbox"/> No				\$270.00	\$135.00	\$
Petition for Extension of Time Fee (3_ months)						\$445.00
OTHER FEES _____ (specify)						\$
TOTAL FEES =						\$481.00

- ☒ Conditional Petition for Extension of Time: An extension of time is requested to provide for timely filing if an extension of time is still required after all papers filed with this communication have been considered.
- ☐ A check in the amount of \$ _____ to cover the above fees is enclosed.
- ☒ Please charge Deposit Account No. 50-1189, Docket No. 22091-701DIV2, in the amount of \$481.00 to cover the above-fees. *A duplicate copy of this sheet is enclosed.*
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-1189, Docket No. 22091-701DIV2. *A duplicate copy of this sheet is enclosed.*

DATE: August 28, 2001

Respectfully submitted,

By: _____

Carol M. Gruppi

Registration No.: 37,341

McCutchen, Doyle, Brown & Enersen, LLP
 Three Embarcadero Center, Suite 1800
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APPLICATION NO	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO
09/577,246	05/24/2000	John L. Schenk	22091-701 DIV2	5239

7590 11/16/2001

McCutchen Doyle Brown & Enersen LLP
Three Embarcadero Center
San Francisco, CA 94111

EXAMINER

MELLER, MICHAEL V

ART UNIT	PAPER NUMBER
----------	--------------

1651

DATE MAILED: 11/16/2001

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	09/577,246	SCHENK, JOHN L.	
	Examiner	Art Unit	
	Michael V. Meller	1651	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) ☒ Responsive to communication(s) filed on 04 September 2001.

2a) ☒ This action is **FINAL**. 2b) ☐ This action is non-final.

3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4) ☒ Claim(s) 27-50 is/are pending in the application.

4a) Of the above claim(s) 35-37 is/are withdrawn from consideration.

5) ☐ Claim(s) _____ is/are allowed.

6) ☒ Claim(s) 27-34 and 38-50 is/are rejected.

7) ☐ Claim(s) _____ is/are objected to.

8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9) ☐ The specification is objected to by the Examiner.

10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11) ☐ The proposed drawing correction filed on _____ is: a) ☐ approved b) ☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12) ☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a) ☐ All b) ☐ Some * c) ☐ None of:

1. ☐ Certified copies of the priority documents have been received.

2. ☐ Certified copies of the priority documents have been received in Application No. _____.

3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14) ☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15) ☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892) 4) ☐ Interview Summary (PTO-413) Paper No(s) _____

2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948) 5) ☐ Notice of Informal Patent Application (PTO-152)

3) ☒ Information Disclosure Statement(s) (PTO-1449) Paper No(s) Z 6) ☐ Other.

DETAILED ACTION

Election/Restrictions

This application contains claims 35-37, which are drawn to an invention nonelected with traverse in Paper No. 5. A complete reply to the final rejection must include cancelation of nonelected claims or other appropriate action (37 CFR 1.144) See MPEP § 821.01.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

Claim Rejections - 35 USC § 103

Claims 27-34 and 38-50 are rejected under 35 U.S.C. 103(a) as being unpatentable over Seidel et al. '689, Seidel et al. '867, Chandler, Johnson or Spaulding taken with Salisbury et al., Critser et al., or Berman et al.

Applicant argues that the primary references do not teach that sorted sperm may be frozen.

In response to applicant's arguments against the references individually, one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references. See *In re Keller*, 642 F.2d 413, 208

USPQ 871 (CCPA 1981); *In re Merck & Co.*, 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986).

The primary references were relied on to show that a sex selected bovine sperm is known in the art. They were not relied on to show a sperm which is frozen after such a process such as flow cytometry. Thus, they do not need to teach each and every claimed limitation since this rejection is under 35 USC 103 and is a rejection with a combination of references.

Applicant also comments further on Spaulding as stating that Spaulding does not sort the sperm, but it is clear from Spaulding that they do, see abstract. Further, as applicant is aware, "sorting" is a broad term and can encompass many meanings. Also see, example 1 of Spaulding which clearly states that they are "sorting" sperm.

Applicant next attempts to argue that the secondary references do not teach freezing selected sperm. Once again, for the above reasons, the references do not have to. This rejection is under 35 USC 103 and the secondary references are used for establishing that it is well known in the art to freeze sperm and the benefits of doing so. Further, one would want to preserve the sperm if he/she was not going to use it right away.

The new claims are also obvious over the cited references for the same reasons as the other claims.

For these reasons and for the reasons of record, the rejection is maintained.

Conclusion

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire **THREE MONTHS** from the mailing date of this action. In the event a first reply is filed within **TWO MONTHS** of the mailing date of this final action and the advisory action is not mailed until after the end of the **THREE-MONTH** shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than **SIX MONTHS** from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michael V. Meller whose telephone number is 703-308-4230. The examiner can normally be reached on Monday thru Friday: 9:00am-5:30pm.

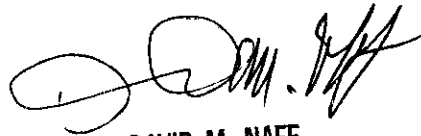
If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Michael Wityshyn can be reached on 703-308-4743. The fax phone numbers for the organization where this application or proceeding is assigned are 703-308-0294 for regular communications and 703-308-0294 for After Final communications.

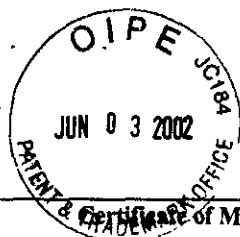
Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is 703-308-0196.

Application/Control Number: 09/577,246
Art Unit: 1651

Page 5

MVM
November 7, 2001


DAVID M. NAFF
PRIMARY EXAMINER
ART UNIT 1651



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Docket No. 22091-701 DIV2

AFI 1651

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<input type="checkbox"/> Pursuant to 37 C.F.R. § 1.6(d), I hereby certify that this paper and all enclosures are being sent via facsimile on the date indicated below to the attention of Examiner _____ at Facsimile No _____ at _____ a.m./p.m.	
Dated: May 16, 2002	Name of Person Certifying: <u>Nancy L. Hug</u> Printed Name: Nancy L. Hug

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John Schenk
Serial No.: 09/577,246
Filing Date: May 24, 2000
Title: METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

Assignee: XY, Inc.
Examiner: M. Meller
Group Art Unit: 1651

Commissioner for Patents
Washington, D.C. 20231

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NOTICE OF APPEAL TO THE BOARD OF PATENT APPEALS
AND INTERFERENCES

Sir:

Applicant hereby appeals to the Board of Appeals from the decision dated November 16, 2001 of the Examiner rejecting claims 27-34 and 38-50. The items checked below are appropriate.

- ☒ Appeal Fee:
- ☐ Large Entity Fee of \$320.00; or
- ☒ Small Entity Fee of \$160.00.
- ☒ Applicant(s) claim Small Entity Status under 37 CFR § 1.27.
- ☒ Petition for Extension of Time (3 mos.)
- ☒ Petition for Extension of Time Fee \$460.00
- ☒ Return Postcard.

06/05/2002 AWONDAF1 00000114 501133 09577246
01 FD:213 160.00 Dk

52078457.1/22091-0701



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Payment of Fees



Enclosed is Check No. _____ in the amount of \$ _____.



Please charge Deposit Account No. 50-1189, Docket No. 22091-701DIV2 in the amount of \$620.00 to cover the above-fees. *A duplicate copy of this sheet is enclosed.*



The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-1189, Docket No. 22091-701DIV2. *A duplicate copy of this sheet is enclosed.*

DATE: May 16, 2002

Respectfully submitted,

By: _____

Carol M. Gruppi

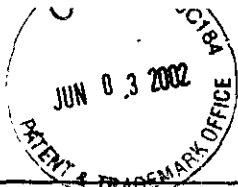
Registration No.: 37,341

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Certificate of Mailing (37 C.F.R. § 1.8(a)).

[X] Pursuant to 37 C.F.R. § 1.8, I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Commissioner for Patents, Washington D.C. 20231.

Dated: May 16, 2002

Name of Person Certifying: Nancy L. Hug
Printed Name: Nancy L. Hug

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John Schenk
Serial No.: 09/577,246
Filing Date: May 24, 2000
Title: Method of Cryopreserving Selected Sperm Cells

Assignee: XY, Inc.
Examiner: M. Meller
Group Art Unit: 1651

Commissioner for Patents
Washington, D.C. 20231

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JUN 11 2002
TECH CENTER 1600/2900

PETITION AND FEE FOR EXTENSION OF TIME
(37 C.F.R. § 1.136(a))

Sir:

Pursuant to 37 C.F.R. § 1.136(a), Applicant hereby petitions for a three month extension of time to respond to the Office Action mailed on November 16, 2001.

1. The communication in connection with the matter for which this extension is requested

- a. ☒ is filed herewith; or
b. ☐ has been filed on _____.

2. ☒ Applicant(s) claim Small Entity Status under 37 CFR § 1.27.

06/05/2002 AWDNAF1 00000114 501139 03577246

02 FD:217 460.00 24

52078460_1

3. The following fees are submitted:

	TOTAL MONTHS REQUESTED	OTHER THAN SMALL ENTITY	SMALL ENTITY	CALCULATIONS
a. <input type="checkbox"/>	one month	\$110.00	\$55.00	\$
b. <input type="checkbox"/>	two month	\$400.00	\$200.00	\$
c. <input checked="" type="checkbox"/>	three month	\$920.00	\$460.00	\$460.00
d. <input type="checkbox"/>	four month	\$1,390.00	\$695.00	\$
e. <input type="checkbox"/>	five month	\$1,890.00	\$945.00	\$
f. <input type="checkbox"/>	An extension for ____ months has already been secured for filing the above-identified communication and the fee paid therefor of \$____ is deducted from the total fee due for the total months of extension now requested. The fee for this extension (\$____), minus the fee previously paid (\$____) equals \$____ (total fee due).			
TOTAL FEES =				\$460.00

- ☒ Conditional Petition for Extension of Time: An extension of time is requested to provide for timely filing if an extension of time is still required after all papers filed with this communication have been considered.
- ☐ A check in the amount of \$ _____ to cover the above fees is enclosed.
- ☒ Please charge Deposit Account No. 50-1189, Docket No. 22091-701DIV2, in the amount of \$460.00 to cover the above-fees.
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-1189, Docket No. 22091-701DIV2.

DATE: May 16, 2002

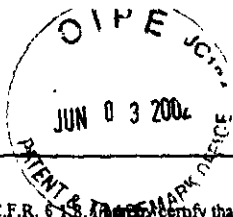
Respectfully submitted,

By:


Carol M. Gruppi

Registration No.: 37,341

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center, Suite 1800
San Francisco, California 94111
Telephone: (650) 849-4902
Telefax: (650) 849-4800



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ORIGINALLY FILED

Docket No. 22091-701DIV2

Certificate of Mailing (37 C.F.R. § 1.8(a)):

[X] Pursuant to 37 C.F.R. § 1.8(a), I hereby certify that this paper and all enclosures are being deposited with the United States Postal Service as first class mail on the date indicated below in an envelope addressed to the Commissioner for Patents, Washington D.C. 20231.

Dated: May 16, 2002

Name of Person Certifying: Nancy L. Hug
Printed Name: Nancy L. Hug

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	John Schenk	Assignee:	XY, Inc.
Serial No.:	09/577,246	Examiner:	M. Meller
Filing Date:	May 24, 2000	Group Art Unit:	1651
Title:	Method of Cryopreserving Selected Sperm Cells		

Commissioner for Patents
Washington, D.C. 20231

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PETITION AND FEE FOR EXTENSION OF TIME
(37 C.F.R. § 1.136(a))

Sir:

Pursuant to 37 C.F.R. § 1.136(a), Applicant hereby petitions for a three month extension of time to respond to the Office Action mailed on November 16, 2001.

1. The communication in connection with the matter for which this extension is requested
 - a. ☒ is filed herewith; or
 - b. ☐ has been filed on _____.

2. ☒ Applicant(s) claim Small Entity Status under 37 CFR § 1.27.



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Docket No. 22091-701DIV2

3. The following fees are submitted: _____

	TOTAL MONTHS REQUESTED	OTHER THAN SMALL ENTITY	SMALL ENTITY	CALCULATIONS
a. <input type="checkbox"/>	one month	\$110.00	\$55.00	\$
b. <input type="checkbox"/>	two month	\$400.00	\$200.00	\$
c. <input checked="" type="checkbox"/>	three month	\$920.00	\$460.00	\$460.00
d. <input type="checkbox"/>	four month	\$1,390.00	\$695.00	\$
e. <input type="checkbox"/>	five month	\$1,890.00	\$945.00	\$
f. <input type="checkbox"/>	An extension for ____ months has already been secured for filing the above-identified communication and the fee paid therefor of \$ ____ is deducted from the total fee due for the total months of extension now requested. The fee for this extension (\$ ____), minus the fee previously paid (\$ ____) equals \$ ____ (total fee due).			
TOTAL FEES =				\$460.00

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- ☒ **Conditional Petition for Extension of Time:** An extension of time is requested to provide for timely filing if an extension of time is still required after all papers filed with this communication have been considered.
- ☐ A check in the amount of \$ _____ to cover the above fees is enclosed.
- ☒ Please charge Deposit Account No. 50-1189, Docket No. 22091-701DIV2, in the amount of \$460.00 to cover the above-fees.
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-1189, Docket No. 22091-701DIV2.

DATE: May 16, 2002

Respectfully submitted,

By:


Carol M. Gruppi

Registration No.: 37,341

McCutchen, Doyle, Brown & Enersen, LLP
Three Embarcadero Center, Suite 1800
San Francisco, California 94111
Telephone: (650) 849-4902
Telefax: (650) 849-4800



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9/19/02

CERTIFICATE OF MAILING

I hereby certify that this paper or fee is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231 on the dated indicated below.

Dated: 8-12-02

Mary R. Zimmerman
Mary R. Zimmerman

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application for:

John L. SCHENK

Serial No.: 09/577,246

Filing Date: May 24, 2000

For: METHOD OF CRYOPRESERVING
SELECTED SPERM CELLS

Examiner: Michael V. Meller

Group Art Unit: 1651

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**REVOCATION OF POWER OF ATTORNEY
AND POWER OF ATTORNEY AND
CHANGE OF CORRESPONDENCE ADDRESS**

Commissioner for Patents
Washington, D.C. 20231

Sir:

XY, Inc., the assignee of record of the entire right, title and interest in the above-identified application, hereby revokes all previous Powers of Attorney and appoints Craig Miles, Reg. No. 45,954 whose mailing address is Santangelo Law Offices, P.C., 125 South Howes Street, 3rd Floor, Fort Collins, CO 80521 as its attorney to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected therewith.

52084309.1/22091-0701

Please direct all telephone calls regarding this application to Craig Miles at (970) 224-3100 and send all correspondence to:

Craig Miles
Santangelo Law Offices, P.C.
125 South Howes, 3rd Floor
Fort Collins, CO 80521
Telephone: (970) 224-3100

Respectfully submitted,

XY, Inc.

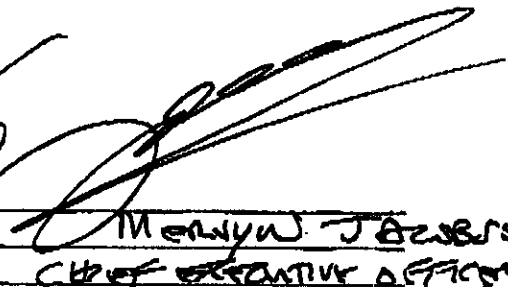
Dated: _____

AUGUST 2nd, 2002.

By: _____

Name: _____

Title: _____


Maryn Johnson
Chief Executive Officer



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STATEMENT UNDER 37 CFR 3.73(b)

Applicant/Patent Owner: John L. SCHENK
Application No./Patent No.: 09/577,246 (a divisional of 09/478,299) Filed/Issue Date: January 5, 2000

XY, INC., a Corporation

(Name of Assignee)

(Type of Assignee, e.g. corporation, partnership, university, government agency, etc.)

states that it is:

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1. ☒ the assignee of the entire right, title and interest; or
2. ☐ an assignee of less than the entire right, title and interest.
The extent (by, percentage) of its ownership interest is _____%.

in the patent application/patent identified above by virtue of either:

- A. ☒ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 010503, Frame 0068, or for which a copy thereof is attached.

OR

- B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as shown below:

1. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at Reel *, Frame *, or for which a copy thereof is attached.

2. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at Reel *, Frame *, or for which a copy thereof is attached.

3. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at Reel *, Frame *, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet.

- ☐ Copies of assignments or other documents in the chain of title are attached.

[NOTE: A separate copy (i.e., the original assignment document or a true copy of the original document) must be submitted to Assignment Division in accordance with 37 CFR Part 3, if the assignment is to be recorded in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

Date

Mervyn Jacobson
Typed or Printed Name

[Signature]
Signature

Chief Executive Officer
Title

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231

52084315.1/22081-0701



UNITED STATES PATENT AND TRADEMARK OFFICE

COMMISSIONER FOR PATENTS
UNITED STATES PATENT AND TRADEMARK OFFICE
WASHINGTON, D.C. 20231
www.uspto.gov

APPLICATION NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTY DOCKET NO /TITLE
09/577,246	05/24/2000	John L. Schenk	22091-701 DIV2

McCutchen Doyle Brown & Enersen LLP
Three Embarcadero Center
San Francisco, CA 94111

CONFIRMATION NO. 5239



OC000000008819973

Date Mailed: 09/19/2002

NOTICE REGARDING POWER OF ATTORNEY

This is in response to the Power of Attorney filed 08/20/2002.

- The Power of Attorney to you in this application has been revoked by the assignee who has intervened as provided by 37 CFR 3.71. Future correspondence will be mailed to the new address of record(37 CFR 1.33).

DORIS M BURNS
1600 (703) 306-0435

OFFICE COPY



10-10-02

\$71650
L

Express Mail No.: EV168806804US
Attorney Docket: XY Frozen Cont

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

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OCT 15 2002

TECH CENTER 1600/2900

#13
Mg
10/22/02

In Re the Application of: John L. Schenk
Serial Number:
Filed:
Parent Title: Method of Cryopreserving Selected Sperm Cells
New Title: Cryopreservation of Selected Sperm Cells
Group Art Unit:
Examiner:
Assignee: XY, Inc.

PETITION FOR EXTENSION OF TIME UNDER 37 C.F.R. § 1.136(a)

Box Patent Application
Commissioner of Patents and Trademarks
Washington, D.C. 20231

Dear Sir:

On behalf of the applicant, the undersigned attorney hereby requests an extension of time pursuant to 37 C.F.R. § 1.136(a) and 37 C.F.R. § 1.17(a)(3). This request is submitted in relation to a Notice of Appeal mailed May 16, 2002, for application number 09/577,246, having two months to file an Appeal Brief or Continuation Application. The Continuation Application would otherwise be due July 16, 2002; however, it is requested that this time period be extended to and

10/11/2002 EFLORES 00000001 09577246

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460.00 OP

including October 16, 2002. This extension will thereby encompass an additional three months extension of time available pursuant to 37 C.F.R. § 1.136(a). Pursuant to 37 C.F.R. §1.17(a)(3), an extension fee of \$460.00 is included with this Petition and the accompanying Continuation Application. In the event the fee has been inappropriately calculated, please allow the maximum extension possible and inform me at your earliest convenience so that any deficiency or error can be corrected immediately.

Dated this 7 day of October, 2002.

Respectfully submitted,
SANTANGELO LAW OFFICES, P.C.

By:


Craig R. Miles
ATTORNEY FOR APPLICANT
PTO No. 45,954
125 South Howes, Third Floor
Fort Collins, CO 80521
(970) 224-3100

Pet3mo-extn



Express Mail No.: EV168806804US
Attorney Docket: XY Frozen Cont

IN THE UNITED STATES PATENT AND
TRADEMARK OFFICE

RECEIVED

OCT 15 2002

TECH CENTER 1600/2900

In Re the Application of: John L. Schenk

Serial Number:

Filed:

Parent Title: Method of Cryopreserving Selected Sperm Cells

New Title: Cryopreservation of Selected Sperm Cells

Group Art Unit:

Examiner:

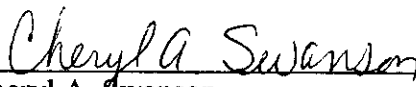
Assignee: XY, Inc.

CERTIFICATE OF EXPRESS MAILING

I, Cheryl A. Swanson, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.
2. I have this day deposited the attached a Petition for Extension of Time with the United States Postal Service as Express Mail, postage prepaid, in an envelope addressed to: The Commissioner of Patents and Trademarks, Washington, D.C. 20231.

Dated this 7 day of October, 2002.


Cheryl A. Swanson



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/577,246	05/24/2000	John L. Schenk	22091-701 DIV2	5239

7590 02/19/2003

CRAIG MILES
SANTANGELO LAW OFFICES P C
125 SOUTH HOWES 3RD FLOOR
FORT COLLINS, CO 80521

EXAMINER

MELLER, MICHAEL V

ART UNIT PAPER NUMBER

1654

DATE MAILED 02/19/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

Notice of Abandonment	Application No.	Applicant(s)	
	09/577,246	SCHENK, JOHN L.	
	Examiner	Art Unit	
	Michael V. Meller	1654	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

This application is abandoned in view of:

1. ☒ Applicant's failure to timely file a proper reply to the Office letter mailed on 16 November 2001.
 - (a) ☐ A reply was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply (including a total extension of time of _____ month(s)) which expired on _____.
 - (b) ☐ A proposed reply was received on _____, but it does not constitute a proper reply under 37 CFR 1.113 (a) to the final rejection.
(A proper reply under 37 CFR 1.113 to a final rejection consists only of: (1) a timely filed amendment which places the application in condition for allowance; (2) a timely filed Notice of Appeal (with appeal fee); or (3) a timely filed Request for Continued Examination (RCE) in compliance with 37 CFR 1.114).
 - (c) ☐ A reply was received on _____ but it does not constitute a proper reply, or a bona fide attempt at a proper reply, to the non-final rejection. See 37 CFR 1.85(a) and 1.111. (See explanation in box 7 below).
 - (d) ☒ No reply has been received.
2. ☐ Applicant's failure to timely pay the required issue fee and publication fee, if applicable, within the statutory period of three months from the mailing date of the Notice of Allowance (PTOL-85).
 - (a) ☐ The issue fee and publication fee, if applicable, was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the statutory period for payment of the issue fee (and publication fee) set in the Notice of Allowance (PTOL-85).
 - (b) ☐ The submitted fee of \$_____ is insufficient. A balance of \$_____ is due.
The issue fee required by 37 CFR 1.18 is \$_____. The publication fee, if required by 37 CFR 1.18(d), is \$_____.
 - (c) ☐ The issue fee and publication fee, if applicable, has not been received.
3. ☐ Applicant's failure to timely file corrected drawings as required by, and within the three-month period set in, the Notice of Allowability (PTO-37).
 - (a) ☐ Proposed corrected drawings were received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply.
 - (b) ☐ No corrected drawings have been received.
4. ☐ The letter of express abandonment which is signed by the attorney or agent of record, the assignee of the entire interest, or all of the applicants.
5. ☐ The letter of express abandonment which is signed by an attorney or agent (acting in a representative capacity under 37 CFR 1.34(a)) upon the filing of a continuing application.
6. ☐ The decision by the Board of Patent Appeals and Interference rendered on _____ and because the period for seeking court review of the decision has expired and there are no allowed claims.
7. ☐ The reason(s) below:



Michael V. Meller
Primary Examiner
Art Unit: 1654

Petitions to revive under 37 CFR 1.137(a) or (b), or requests to withdraw the holding of abandonment under 37 CFR 1.181, should be promptly filed to minimize any negative effects on patent term.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

REQUEST FOR ACCESS TO AN ABANDONED APPLICATION UNDER 37 CFR 1.14

Bring completed form to:
File Information Unit
Crystal Plaza Three, Room 1D01
2021 South Clark Place
Arlington, VA
Telephone: (703) 308-2733

In re Application of
Schenk

Application Number
09/577246

Filed
5/24/00

Paper No. **15**

I hereby request access under 37 CFR 1.14(a)(1)(iv) to the application file record of the above-identified **ABANDONED** application, which is identified in, or to which a benefit is claimed, in the following document (as shown in the attachment):

United States Patent Application Publication No. **20030157475**, page, _____ line _____,

United States Patent Number _____, column _____, line, _____ or

WIPO Pub. No. _____, page _____, line _____.

Related Information about Access to Pending Applications (37 CFR 1.14):

Direct access to pending applications is not available to the public (see 37 CFR 1.14(c) if applicant) but copies may be available and may be purchased from the Office of Public Records upon payment of the appropriate fee (37 CFR 1.19(b)), as follows:

For published applications that are still pending, a member of the public may obtain a **copy** of:

- the file contents;
- the pending application as originally filed; or
- any document in the file of the pending application.

For unpublished applications that are still pending:

- (1) If the **benefit of the pending application is claimed** under 35 U.S.C. 119(e), 120, 121, or 365 in another application that has: (a) issued as a U.S. patent, or (b) published as a statutory invention registration, a U.S. patent application publication, or an international patent application publication in accordance with PCT Article 21(2), a member of the public may obtain a **copy** of:
 - the file contents;
 - the pending application as originally filed; or
 - any document in the file of the pending application.
- (2) If the application is **incorporated by reference or otherwise identified** in a U.S. patent, a statutory invention registration, a U.S. patent application publication, or an international patent application publication in accordance with PCT Article 21(2), a member of the public may obtain a **copy** of:
 - the pending application as originally filed.

Burt Murphy

Signature

Burt Murphy, Patent Gateway

Typed or printed name

Registration Number, if applicable

866.728.4283

Telephone Number

September 13, 2004

Date

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Approved by: _____

Unit: _____

SEP 13 2004

File Information Unit

This collection of information is required by 37 CFR 1.14. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **BRING TO: File Information Unit, Crystal Plaza Three, Room 1D01, 2021 South Clark Place, Arlington, VA.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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REQUEST FOR ACCESS TO AN ABANDONED APPLICATION UNDER 37 CFR 1.14

Bring completed form to:

File Information Unit, Suite 3A20
2800 South Randolph Street
Arlington, VA 22206

Telephone: (703) 756-1800 x 3 2010

In re Application of

Application Number

09/577 246

Filed

5-24-00

Paper No.

#16

File Information Unit

I hereby request access under 37 CFR 1.14(a)(1)(iv) to the application file record of the above-identified ABANDONED application, which is not within the file jacket of a pending Continued Prosecution Application (CPA) (37 CFR 1.53(d)) and which is identified in, or to which a benefit is claimed, in the following document (as shown in the attachment):

United States Patent Application Publication No. 2003/0157475, page, line

United States Patent Number, column, line,

WIPO Pub. No., page, line

Related Information About Access to Applications Maintained in the Image File Wrapper System (IFW) and Access to Pending Applications in General

A member of the public, acting without a power to inspect, cannot order applications maintained in the IFW system through the FIU. If the member of the public is entitled to a copy of the application file, then the file is made available through the Public Patent Application Information Retrieval system (Public PAIR) on the USPTO internet web site (www.uspto.gov). Terminals that allow access to Public PAIR are available in the Public Search Room. The member of the public may also be entitled to obtain a copy of all or part of the application file upon payment of the appropriate fee. Such copies must be purchased through the Office of Public Records upon payment of the appropriate fee (37 CFR 1.19(b)).

For published applications that are still pending, a member of the public may obtain a copy of:

the file contents; the pending application as originally filed; or any document in the file of the pending application.

For unpublished applications that are still pending:

- (1) If the benefit of the pending application is claimed under 35 U.S.C. 119(e), 120, 121, or 365 in another application that has: (a) issued as a U.S. patent, or (b) published as a statutory invention registration, a U.S. patent application publication, or an international patent application publication in accordance with PCT Article 21(2), a member of the public may obtain a copy of the file contents; the pending application as originally filed, or any document in the file of the pending application
- (2) If the application is incorporated by reference or otherwise identified in a U.S. patent, a statutory invention registration, a U.S. patent application publication, or an international patent application publication in accordance with PCT Article 21(2), a member of the public may obtain a copy of the pending application as originally filed.

Signature

Ingrid Arbuckle

Typed of printed name

Registration Number, if applicable

Telephone Number

Date

12-13-10

Approved by

Unit

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(Initials) 0000

F.I.U.

This collection of information is required by 37 CFR 1.11 and 1.14. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. BRING TO: File Information Unit, Suite 3A20, 2800 South Randolph Street, Arlington, Virginia.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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USPT,JPAB,EPAB,DWPI,TDBD	sex	53646	L6
USPT,JPAB,EPAB,DWPI,TDBD	select or selected or selecting or sort or sorted or sorting	2382115	L5
USPT,JPAB,EPAB,DWPI,TDBD	bovine same 13	53	L4
USPT,JPAB,EPAB,DWPI,TDBD	11 same 12	719	L3
USPT,JPAB,EPAB,DWPI,TDBD	freeze or frozen or freezing or freezes	191674	L2
USPT,JPAB,EPAB,DWPI,TDBD	sperm	10618	L1

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USPT	5514537.pn. and l11	1	L28
USPT	l26 and l11	0	L27
USPT	5135759.pn.	1	L26
USPT	l11 and l24	1	L25
USPT	5021244.pn.	1	L24
USPT	5021244	13	L23
USPT,JPAB,EPAB,DWPI,TDBD	5021244	20	L22
USPT,JPAB,EPAB,DWPI,TDBD	l17 and l18 and l20	25	L21
USPT,JPAB,EPAB,DWPI,TDBD	sort or sorted or sorting	143747	L20
USPT,JPAB,EPAB,DWPI,TDBD	sort or sorted	116983	L19
USPT,JPAB,EPAB,DWPI,TDBD	select or selected	2184874	L18
USPT,JPAB,EPAB,DWPI,TDBD	sperm and sex and l9 and l11	58	L17
USPT,JPAB,EPAB,DWPI,TDBD	l15 same l9 same l11	0	L16
USPT,JPAB,EPAB,DWPI,TDBD	sex	53645	L15
USPT,JPAB,EPAB,DWPI,TDBD	l11 same sperm same l9	3	L14
USPT,JPAB,EPAB,DWPI,TDBD	l11 and sperm and l9	302	L13
USPT,JPAB,EPAB,DWPI,TDBD	l11 and l10	3	L12
USPT,JPAB,EPAB,DWPI,TDBD	freeze or freezing or frozen	191619	L11
USPT,JPAB,EPAB,DWPI,TDBD	sex selected	25	L10
USPT,JPAB,EPAB,DWPI,TDBD	flow cytometry	4461	L9
USPT,JPAB,EPAB,DWPI,TDBD	l7 same l6	4	L8
USPT,JPAB,EPAB,DWPI,TDBD	impurity or impurities	329749	L7
USPT,JPAB,EPAB,DWPI,TDBD	l5 same l4	120	L6
USPT,JPAB,EPAB,DWPI,TDBD	purif\$6	329664	L5
USPT,JPAB,EPAB,DWPI,TDBD	l2 same l3	676	L4
USPT,JPAB,EPAB,DWPI,TDBD	polyol or sorbitol or glycerol	191150	L3
USPT,JPAB,EPAB,DWPI,TDBD	l1 same enzyme	34307	L2
USPT,JPAB,EPAB,DWPI,TDBD	filtering or filter or filtered or filtration or separation or separating or separate or membrane	2977808	L1

PATENT APPLICATION FEE DETERMINATION RECORD

Effective December 29, 1999

Application or Docket Number

0987246

CLAIMS AS FILED - PART I

(Column 1) (Column 2)

FOR	NUMBER FILED	NUMBER EXTRA
BASIC FEE		
TOTAL CLAIMS	11 minus 20=	*
INDEPENDENT CLAIMS	1 minus 3 =	*
MULTIPLE DEPENDENT CLAIM PRESENT		

* If the difference in column 1 is less than zero, enter "0" in column 2

SMALL ENTITY
TYPE ☐

OR

OTHER THAN
SMALL ENTITY

RATE	FEE
	345.00
X\$ 9=	
X39=	
+130=	
TOTAL	

OR

RATE	FEE
	690.00
X\$18=	
X78=	
+260=	
TOTAL	100

CLAIMS AS AMENDED - PART II

(Column 1) (Column 2) (Column 3)

AMENDMENT A	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA
Total	*	Minus **	=
Independent	*	Minus ***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM			

SMALL ENTITY

OR

OTHER THAN
SMALL ENTITY

RATE	ADDI- TIONAL FEE
X\$ 9=	
X39=	
+130=	
TOTAL ADDIT. FEE	

OR

RATE	ADDI- TIONAL FEE
X\$18=	
X78=	
+260=	
TOTAL ADDIT. FEE	

(Column 1) (Column 2) (Column 3)

AMENDMENT B	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA
Total	* 24	Minus ** 20	= 4
Independent	* 2	Minus *** 3	= 0
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM			

RATE	ADDI- TIONAL FEE
X\$ 9=	360.00
X39=	
+130=	
TOTAL ADDIT. FEE	360.00

OR

RATE	ADDI- TIONAL FEE
X\$18=	
X78=	
+260=	
TOTAL ADDIT. FEE	

(Column 1) (Column 2) (Column 3)

AMENDMENT C	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA
Total	*	Minus **	=
Independent	*	Minus ***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM			

RATE	ADDI- TIONAL FEE
X\$ 9=	
X39=	
+130=	
TOTAL ADDIT. FEE	

OR

RATE	ADDI- TIONAL FEE
X\$18=	
X78=	
+260=	
TOTAL ADDIT. FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20."

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3."

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET (FOR USE WITH FORM PTO-875)						SERIAL NO.		FILING DATE			
						APPLICANT(S)					
CLAIMS											
	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT			*	*	*	
	IND.	DEP.	IND.	DEP.	IND.	DEP.		IND.	DEP.	IND.	DEP.
1							51				
2							52				
3							53				
4							54				
5							55				
6							56				
7							57				
8							58				
9							59				
10							60				
11							61				
12							62				
13							63				
14							64				
15							65				
16							66				
17							67				
18							68				
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37							87				
38							88				
39							89				
40							90				
41							91				
42							92				
43							93				
44							94				
45							95				
46							96				
47							97				
48							98				
49							99				
50							100				
TOTAL IND.							TOTAL IND.				
TOTAL DEP.							TOTAL DEP.				
TOTAL CLAIMS							TOTAL CLAIMS				