



Industria y Comercio
SUPERINTENDENCIA

PCT

SOLICITUD

PATENTE DE INVENCION

03-54828

21. EXPEDIENTE Nº

54. TÍTULO DISPOSICIÓN PARA SEPARAR ESPERMATOZOIDES CONGELADOS EN POBLACIONES QUE CONTIENEN
CRÓMOSOMAS X Y CRÓMOSOMAS Y

51. CLASIFICACIÓN INTERNACIONAL A01K 67/00; C12N 5/00

71. SOLICITANTE XY? INC Y COLORADO STATE UNIVERSITY RESEARCH FOUNDATION

DOMICILIO Fort Collins, Colorado, Estados Unidos de America

74. APODERADO Enilio Ferrero
Código 1092

22. BOGOTÁ, D. C.



Industria y Comercio
SUPERINTENDENCIA



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PETITORIO

SUPERINDUSTRIA Y COMERCIO

Radicación : 03054828 00000000

Folios: 120

Fecha (HUB): 2003-06-27 17:52:13

Trámite : 011 PCT-PATENT 379 FASE NACI 411 PRESENTAC

Dependencia: 2000 DIVISION DE NUEVAS CREACIONES

FORMULARIO UNICO DE SOLICITUD DE PATENTE
PCT
ENTRADA EN FASE NACIONAL

1

SOLICITUD DE:



Patente de Invención.



Patente de Modelo de Utilidad.



Capítulo I.



Capítulo II.

2

SOLICITANTE
(71)

Nombre: XY, INC. Y COLORADO STATE
UNIVERSITY, por medio de su agente,
COLORADO STATE UNIVERSITY
RESEARCH FOUNDATION

sociedades constituidas y existentes bajo
las leyes del estado de Colorado,
Estados Unidos de América

Dirección: 1108 North Lemay Avenue,
Fort Collins, Colorado 80524,
Estados Unidos de América y

Room 410, 801 South Howes
Street,
Fort Collins, Colorado 80522
Estados Unidos de América

Domicilio: Fort Collins, Colorado
Estados Unidos de América

IDENTIFICACION

C.C.



NIT



C.E.



Otro



Cual _____

Número: _____

3

REPRESENTANTE
O APODERADO

Nombre: EMILIO FERRERO

Dirección: Carrera 4ª. No. 72-35

Teléfono: 347 38 11

Fax:: 217 92 11

E-mail: clientes@camyp.com.

Domicilio: Bogotá, Colombia.

IDENTIFICACION

C.C.



NIT



C.E.



Otro



Cual _____

Número: 438.019 T.P. 31.929

4

INVENTOR (ES)
(72)

1- George, E. SEIDEL

2 -Kwang Tae SUH

3- Kehuan LU

1,2,3

1108 North Lemay Avenue,
Fort Collins CO 80524,
Estados Unidos de América

5

PCT (86)

Solicitud Internacional No. PCT7US01/45023Publicación Internacional No. WO 02/43574 A2Fecha: 29 de noviembre de 2001Fecha : 8 de junio de 2002

6 Título (54) DISPOSICIÓN PARA SEPARAR ESPERMATOZOIDES CONGELADOS EN POBLACIONES QUE CONTIENEN CROMOSOMAS X y CROMOSOMAS Y

7 Clasificación Internacional (51) A61B; A01K 67/00, 67/033; C12N 5/00, 5/02, 15/00.

8	Prioridad SI ☒ NO ☐	(33) País de Origen	(32) Fecha	(31) Número de Solicitud
		US	29 de noviembre de 2000	60/253.785
		US	29 de noviembre de 2000	60/253.787

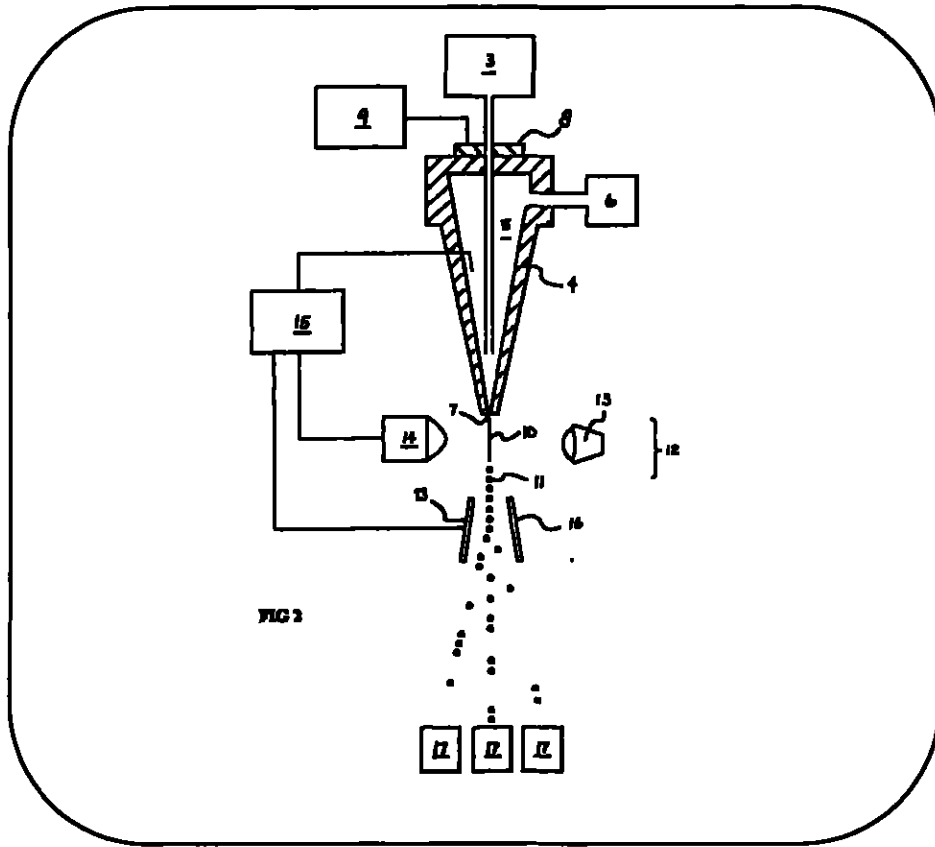
9 Comprobante de pago No.: 03-44.020 Fecha: 24 de junio de 2003

10 Anexos:

- ☒ Comprobante de pago de la tasa de presentación de la solicitud por \$ 400.000
- ☐ Comprobante de pago por reivindicación de prioridad.
- ☒ Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica.
- ☒ Poderes, si fuere el caso.
- ☐ Documento de cesión del inventor al solicitante o a su causante.
- ☐ Declaraciones.
- ☒ Copia de la solicitud internacional. (Resumen, descripción, reivindicaciones, dibujos)
- ☒ Traducción de la solicitud internacional al castellano. (Resumen, descripción, reivindicaciones, dibujos)
- ☐ Copia del examen preliminar efectuado por la IPEA.
- ☐ Traducción del examen preliminar efectuado por la IPEA.
- ☐ De ser el caso, copia del contrato de acceso.
- ☐ De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales de las comunidades indígenas.
- ☐ De ser el caso, certificado de depósito del material biológico.
- ☒ Arte final 12x12
- ☐ De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el mismo titular o su causante, relacionadas parcial o totalmente con la invención de esta solicitud.
- ☐ Otros

11

FIGURA CARACTERISTICA:



12

Solicito la concesión de la patente,

NOMBRE: EMILIO FERRERO

FIRMA: *Emilio Ferrero*

C.C. 438.019 de Usaquén T.P. 31.929

MRE/mrm-ld-

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41013



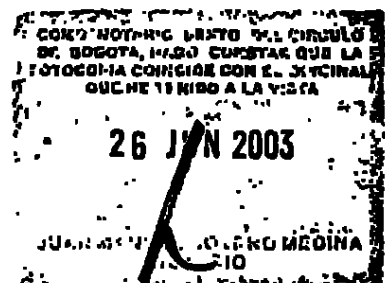
APOSTILLE
(Convention de La Haye du 5 October 1961)

1. Country: United States of America
This public document
2. has been signed by CHERYL A SWANSON
3. acting in the capacity of Notary Public, State of Colorado
4. bears the seal/stamp of the CHERYL A SWANSON, Notary Public, State of Colorado

CERTIFIED

5. at Denver, Colorado
6. this 6th day of May, 2003
7. By DONETTA DAVIDSON, Secretary of State, State of Colorado
8. Certificate Number 67398.
9. (OFFICIAL SEAL)
10. Signature:

Donetta Davidson



Representación y Poder con autenticación notarial y Apostille para patentes, modelos de utilidad, diseños industriales, marcas de fábrica, nombres comerciales, enseñas, nombres de dominio, lemas comerciales, licencias.

Representation and Power-of-Attorney with notarial authentication and Apostille for patents, utility models, industrial designs, trademarks, commercial names, trade emblems, domain names, slogans, licenses.

REPRESENTACION Y PODER

REPRESENTATION AND POWER-OF-ATTORNEY

Yo (nosotros), abajo firmado (s)/I (we), the undersigned

Mervyn Jacobson

domiciliado (s) en/of XY, Inc. Fort Collins, Colorado 80524, Estados Unidos

hereby ratify the patent applications No. 02.101.971 and No. 02.112.918 filed by EMILIO FERRERO on November 8, 2002, and December 12, 2002 respectively, and

por el presente nombramos a EMILIO FERRERO, tpa. 31929; GONZALO PERDOMO, tpa. 831; LUZ CLEMENCIA DE PAEZ, tpa. 23555 y GERMAN CAVELIER, tpa. 832, domiciliados en la Carrera 4a N° 72-35, Bogotá, Colombia, en obediencia a lo dispuesto en el párrafo 1o del Artículo 543 del Código de Comercio, el primero como principal y los demás como suplentes, en su orden, como mis (nuestros) representantes en todos los asuntos de propiedad industrial con facultad para recibir notificaciones y nombrar apoderados judiciales o extrajudiciales. El primer representante suplente reemplazará al principal en caso de ausencia o impedimento temporal o definitivo de aquél. La misma regla se aplicará cuando el segundo representante suplente haya de reemplazar al primero, o el tercero al segundo. En tales casos, el representante principal, o el primero, o segundo suplente, en su caso, o ambos, declararán su intención de no ejercer la representación mediante declaración autenticada por Notario Público en Colombia, o por Notario u otro Funcionario Público, o Cónsul de Colombia, en el extranjero; y si se tratare de impedimento, con el certificado respectivo. Si alguno de los nombrados faltare definitivamente, el siguiente tomará su lugar. Cuando cesare la ausencia o impedimento del representante principal, o de su primero o segundo suplentes, en su caso, podrán ejercer la representación, en su orden, uno u otros según el caso, asumiéndola por el solo hecho de ejercerla, cesando en ese momento el ejercicio de la representación por el primero, segundo o tercer suplente en su caso.

Además, confiero (conferimos) poder a los abogados arriba nombrados, para obtener la protección de mi (nuestra) propiedad industrial y contra la competencia desleal, a cuyo efecto autorizo (amos) a los dichos apoderados para que me (nos) representen ante cualesquiera entidades o personas, ejerzan o no jurisdicción, especialmente ante las del orden administrativo, contencioso-administrativo y judicial, así como los tribunales de arbitramento, en todo lo concerniente a los siguientes asuntos: a) Obtención de patentes de invención, modelos de utilidad y diseños industriales; el registro de marcas de fábrica, colectivas, defensivas, de servicio, lemas comerciales, nombres comerciales,

enseñas, nombres de dominio, variedades vegetales y denominaciones de origen; su renovación, traspaso, cambio de nombre o domicilio, cancelación voluntaria; y el registro de licencias de uso de esos derechos; b) Las acciones de competencia desleal, protección del consumidor, oposiciones u observaciones, cancelación administrativa y judicial, nulidad, medidas cautelares, concesión de licencias, la tutela, la protección de secretos industriales, y en general los juicios civiles, penales, administrativos o contencioso-administrativos relacionados con estos derechos; acciones y procesos que promovamos o se nos

in due compliance with the terms of paragraph 1st. of article 543 of the Commercial Code, do hereby appoint EMILIO FERRERO, tpa. 31929; GONZALO PERDOMO, tpa. 831; LUZ CLEMENCIA DE PAEZ, tpa. 23555 and GERMAN CAVELIER, tpa. 832, domiciled at Carrera 4a N° 72-35, Bogotá, Colombia, the first as principal and the others as alternates, in the order of their appointment, as our representatives for all industrial property matters with faculty to receive notifications and to appoint attorneys in and out of Court. The first alternate shall replace the principal in case of his absence or of temporal or definitive impediment. The same rule will apply when the second alternate representative is to replace the first one, or when the third is to replace the first or second alternates in their turn, or both of them, second. In such cases, the principal representative, or the first or second alternates in their turn, or both of them, shall state their intention not to act as representatives through a declaration given before a Notary Public in Colombia, or before a Notary or another Public Official or a Colombian Consul abroad; and in case of impediment, the appropriate certificate shall suffice. If any of the appointees were to be definitely absent, the following alternate shall take his place. When the absence or impediment of the principal representative, or of the first or second alternate in their turn ceases, the representation can be taken again in their sequence, by the principal, the first or second alternate depending on case, and the mere fact of its exercise will suffice. The exercise of the representation by the first, second or third alternates in their turn shall end at such time. In addition, we grant a Power of Attorney in favor of the above-named attorneys at law so that they may obtain the protection of my (our) industrial property and against the unfair competition, for which purpose I (we) authorize the said attorneys to represent me (us) before any authorities or persons, with or without jurisdiction, specially those administrative, administrative contentious and judicial, as well as before Arbitration Court, in all matters concerning the following: a) Obtainment of patents, utility models and industrial designs; the registration of trademarks, collective, defensive and service marks,

slogans, commercial names, trade emblems, domain names, vegetal varieties and denominations of origin; its renewal, assignment, change of name or domicile, voluntary cancellation; and the registration of licenses of use of the above rights; b) Actions of unfair competition, protection to the consumer, oppositions or observations, administrative or Court cancellation, nullity, infringement, granting of licenses, the action for writ mandamus, the protection of trade secrets, and in general the civil, penal, and administrative suits relating to those rights; actions and suits instituted by me (us) or against me (us). And that in all the above matters

prueben. Y para que en todos los asuntos arriba determinados, puedan
sustituir este mandato, transigir, conciliar, admitir los hechos del
proceso, desistir, cancelar, recibir, renunciar, y ratificar los actos de
agentes oficiosos.

they may substitute this Power of Attorney, compromise, conciliate,
admit the facts of the case, desist, cancel, receive, resign, and ratify acts
done by representatives without a power of attorney.

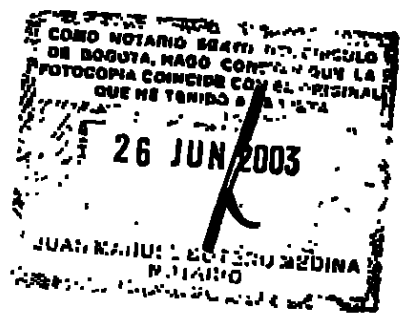
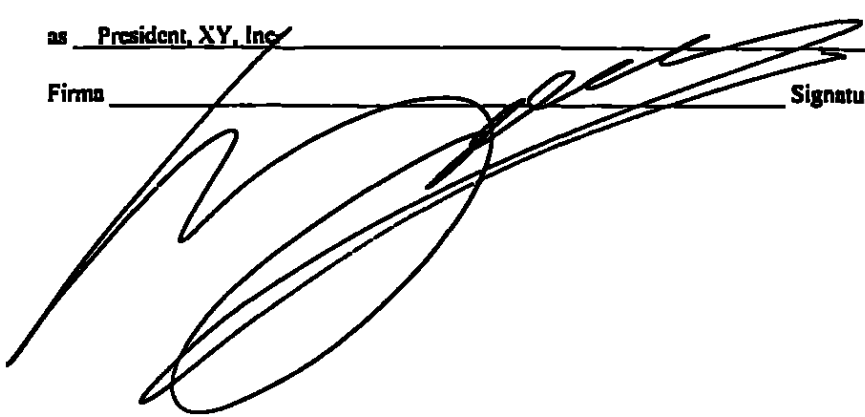
Dado y firmado hoy/Given and signed this 3^d day of FEB, 2003

en/in Fort Collins, Colorado

por/by Mervyn Jacobson

as President, XY, Inc

Firma _____ Signature



CERTIFICADO NOTARIAL (INDIVIDUO)

A los 3 días del mes de Febrero
de 2003 ante mí, Notario Público o Funcionario Autorizado
del Estado de Colorado EEUU
compareció(eron) personalmente _____

a quien(es) doy fe de conocer y me consta que es(son) la(s)
persona(s) firmante(s) del documento que precede, y quien(es)
tiene(n) capacidad legal para otorgarlo

Notario Público o Funcionario Autorizado -- Notary Public or Attesting Official

CERTIFICADO NOTARIAL (SOCIEDAD)

Hoy 3 de FEBRERO de 2003
Compareció(eron) ante mí
Mervyn Jacobson
quien(es) conozco personalmente y que firma(n) este
documento en representación de la Sociedad/compañía
denominada XY, Inc.

Certifico:

1. Que he visto los siguientes documentos:*

Informe de la Sociedad or Compañía de
Responsabilidad Limitada fechado 20 Junio,
1998, presentado con el Secretario de Estado
del Estado de Colorado, lo cual es una copia
fidel y correcta obtenida de www.sos.state.co.us
y leído por el suscrito

2. Que dichos documentos prueban lo siguiente:

2.1. Que XY, Inc.

es una Sociedad/Compañía legalmente constituida y existente
de acuerdo con las leyes del Estado de Colorado,
Estados Unidos de America

2.2. Que el domicilio de dicha Sociedad/Compañía es
1108 North Lemay Avenue, Fort Collins,
Colorado 80524

2.3. Que el objeto u objetos para los cuales se otorga el presente
poder están comprendidos dentro del objeto social de la
mencionada Sociedad/Compañía.

2.4. Que el(los) Sr.(Sres.) Mervyn Jacobson, Presidente
de XY, Inc.
está(n) autorizado(s) para otorgar el presente Poder.

Ciudad Fort Collins

Estado Colorado, Estados Unidos de America

Hoy 3 Febrero de 2003

Notario Público o Funcionario Autorizado -- Notary Public or Attesting Official

* Los documentos deben identificarse con fecha y origen

CAVELIER ABOGADOS
WFFODE01-181 (MINUTA Nº12.1.1.2.6 USA)

NOTARIAL CERTIFICATE (INDIVIDUAL)

On this 3 day of February
2003 before me, a Notary Public or Attesting Official of
the State of Colorado USA
personally appeared Mervyn Jacobson
to me known, and known to me to be the person(s) who
executed that foregoing document, and who (have) legal
capacity to execute it.

My Commission Expires 08-21-2003

NOTARIAL CERTIFICATE (CORPORATION)

My Commission Expires 08-21-2003

On this 3 day of February 2003
appeared before me
Mervyn Jacobson
who(m) I know personally and who(m) signed this document as
representative(s) of the Corporation/Company named:
XY, Inc.

I certify:

1. That I have seen the following documents:*

Biennial Report of Corporation or Limited Liability
Company, dated June 20, 1998, and filed with the
Colorado Secretary of States office, a true and correct
copy of which was downloaded from
www.sos.state.co.us and read by the undersigned.

2. That the above documents duly prove the following:

2.1. That XY, Inc.

is a Corporation/Company legally constituted and in existence
in accordance with the laws of the State of Colorado, United
States of America.

2.2. That the domicile of said Corporation/Company is _____

1108 North Lemay Avenue, Fort Collins, Colorado 80524

2.3. That the purpose or purposes for which the present Power
of Attorney is granted are comprised within the corporation
purposes set forth in the charter of the mentioned
Corporation/Company.

2.4. That Mr.(Messrs.) Mervyn Jacobson, President of XY,
Inc. is(are) authorized to grant the present Power of Attorney.

City: Fort Collins

State: Colorado, United States of America

This 3 day of February 2003

Craig R. Miles
Santangelo Law Offices, P.C.

Notary Public or Attesting Official Cheryl A. Sullivan 3/2003
My commission Expires 8/21/2003

* Documents must be identified with date and origin

My Commission Expires 08-21-2003

TRADUCCIÓN OFICIAL NÚMERO 071/2003 REALIZADA POR EDUARDO CALADO NOGUERA, TRADUCTOR OFICIAL APROBADO POR RESOLUCIÓN 541 DEL 27 DE FEBRERO DE 1976 Y DEBIDAMENTE INSCRITO ANTE EL MINISTERIO DE RELACIONES EXTERIORES DE COLOMBIA, DE LOS SELLOS Y AUTENTICACIONES QUE APARECEN EN EL DOCUMENTO DE REPRESENTACIÓN, RATIFICACIÓN Y PODER OTORGADO POR MERVYN JOHNSON A NOMBRE DE XY INC, DOMICILIADA EN FORT COLLINS, COLORADO 80524, ESTADOS UNIDOS DE AMÉRICA, POR EL CUAL RATIFICAN LAS SOLICITUDES DE PATENTE NÚMEROS 02.101.971 Y 02.112.918 , PRESENTADAS POR EL DOCTOR EMILIO FERRERO LOS DIAS 8 DE NOVIEMBRE DE 2002 Y 12 DE DICIEMBRE DE 2002, RESPECTIVAMENTE Y QUE IGUALMENTE OTORGA PODER A LOS DOCTORES EMILIO FERRERO, TPA 31929, GONZALO PERDOMO, TPA 831, LUZ CLEMENCIA DE PAEZ, TPA 23555 Y GERMÁN CAVELIER, TPA 832. Dado y firmado hoy 3 de febrero de 2003 en Fort Collins, Colorado, Mervyn Jacobson como presidente de XY, Inc. Firmado: ilegible.

Certificado notarial de fecha 3 de febrero de 2003 por el cual se certifica la comparecencia de Mervyn Jacobson ante mi, notario público o funcionario autorizado del estado de Colorado, Estados Unidos de América. Firmado: Cheryl A. Swanson.

Sello: Cheryl A. Swanson, notario público, estado de Colorado.

Certificado notarial en inglés y en español, de fecha 3 de febrero de 2003 para certificar la comparecencia de Mervyn Jacobson como representante de XY, Inc., la existencia de la empresa y su domicilio y la capacidad de dicha persona para otorgar el presente poder. Ciudad : Fort Collins, Colorado, Estados Unidos de America, 3 de febrero de 2003. Firmado: Craig R. Miles, Santangelo Law Offices, P.C.

Firmado: Cheryl A. Swanson, 3 de febrero de 2003.

Sello: Estado de Colorado, 1876

APOSTILLA:

(Convención de La Haya del 5 de octubre de 1961)

1. Estados Unidos de América

Este documento público

2. ha sido firmado por CHERYL A. SWANSON

3. quien actúa en su calidad de NOTARIO PÚBLICO, ESTADO DE COLORADO

4. lleva el sello de CHERYL A. SWANSON, NOTARIO PÚBLICO, ESTADO DE COLORADO

CERTIFICADO

5. en Denver, Colorado

6. el 6 de mayo de 2003

7. por DONETTA DAVIDSON, Secretario de estado, estado de Colorado.

8. Número 67398

9. Sello oficial

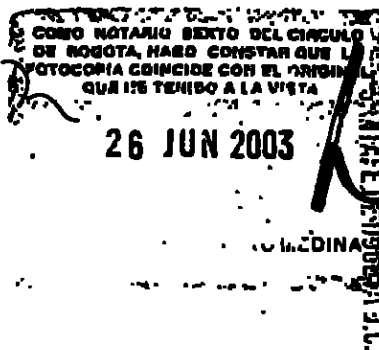
10. Firma: DONETTA DAVIDSON

COLO-13534-841-001-4

WPCL002G ID 4468

Bogotá, 20 de mayo de 2003.

Eduardo Calado
Eduardo Calado Negrete
•TRADUCTOR OFICIAL
INGLÉS - ESPAÑOL
Res 541 febrero de 1997



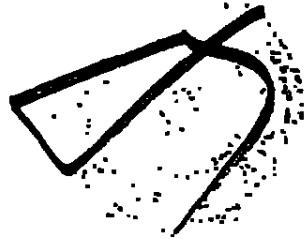
445150
Eduardo Calado
LAS FOTOCOPIAS DE LAS VISTAS

MINISTERIO DE EL AGROPECUARIAS

MINISTERIO DE EL AGROPECUARIAS

2003 MAY 21 10:13

CO. CAFFE DE
IES DE
*Palacio de
Café de
Café de*



COMO MANTENEDOR DEL CIRCULO
DE FERIA. MANTENEDOR QUE LA
FERIA SE CONDUCE CON EL OFICIAL
QUE SE ENCUENTRA A LA VISTA

23 JUN 2003

LOHIA

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
6 June 2002 (06.06.2002)

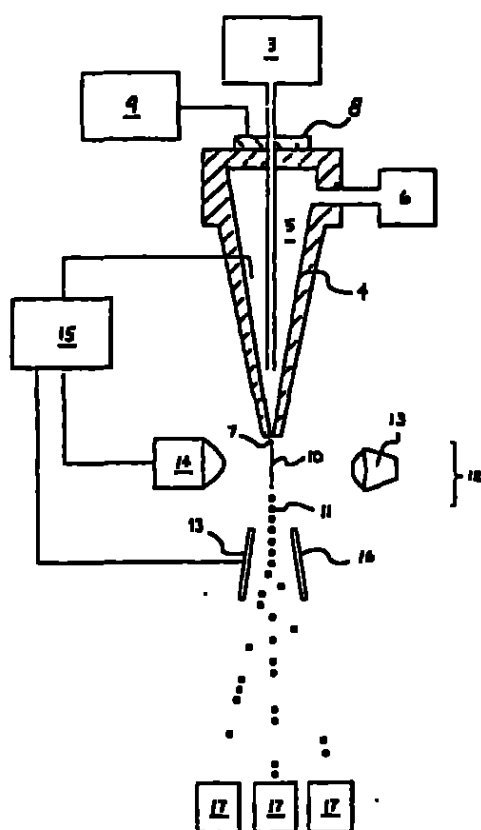
PCT

(10) International Publication Number
WO 02/43574 A2

- (51) International Patent Classification: A61B US 60/253,787 (CON)
Filed on 29 November 2000 (29.11.2000)
- (21) International Application Number: PCT/US01/45023
- (22) International Filing Date: 29 November 2001 (29.11.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/253,785 29 November 2000 (29.11.2000) US
60/253,787 29 November 2000 (29.11.2000) US
- (63) Related by continuation (CON) or continuation-in-part (CIP) to earlier applications:
US 60/253,785 (CON)
Filed on 29 November 2000 (29.11.2000)
- (71) Applicants (for all designated States except US): XY, INC. [US/US]; 1108 North Lemay Avenue, Fort Collins, CO 80524 (US); COLORADO STATE UNIVERSITY, through its agent, COLORADO STATE UNIVERSITY RESEARCH FOUNDATION [US/US]; Room 410, 601 South Howes Street, Fort Collins, CO 80522 (US).
- (72) Inventors: and
- (75) Inventors/Applicants (for US only): SEIDEL, George, E. [US/US]; XY, Inc., 1108 North Lemay Avenue, Fort Collins, CO 80524 (US); SUH, Tae Kwang [US/US]; XY, Inc., 1108 North Lemay Avenue, Fort Collins, CO 80524 (US); LI, Kehuan [US/US]; XY, Inc., 1108 North Lemay Avenue, Fort Collins, CO 80524 (US).

[Continued on next page]

(54) Title: SYSTEM TO SEPARATE FROZEN-THAWED SPERMATOZOA INTO X-CHROMOSOME BEARING AND Y-CHROMOSOME BEARING POPULATIONS



(57) Abstract: Devices, compositions, and methods for handling, separating, packaging, and utilization of spermatozoa (1) that can be derived from previously frozen sperm samples collected from a male mammel. Specifically, techniques to uniformly stain (2) spermatozoal DNA even when derived from previously frozen sperm and separation techniques to separate and isolate spermatozoa even when derived from previously frozen sperm samples into X-chromosome bearing and Y-chromosome bearing populations having high purity.

WO 02/43574 A2

- Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR,
GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent
(BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR,
NE, SN, TD, TG).**

- Declaration under Rule 4.17:**

- of inventorship (Rule 4.17(n)) for US only

Published:

— without international search report and to be republished upon receipt of that report

- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.**

SYSTEM TO SEPARATE FROZEN-THAWED SPERMATOOZOA INTO X-CHROMOSOME BEARING AND Y-CHROMOSOME BEARING POPULATIONS

This application claims the benefit of United States Provisional Patent Application
5 No. 60/253,787, filed November 29, 2000 and United States Provisional Patent Application
No. 60/253,785, filed November 29, 2000, each hereby incorporated by reference herein.

I TECHNICAL FIELD

The invention involves the substantially uniform binding of fluorochrome(s) to the
10 DNA within mammalian spermatozoa (or sperm cells) allowing such labeled spermatozoa to
be separated into high purity X-chromosome bearing and Y-chromosome bearing
populations. Specifically, methods for the substantially uniform binding of fluorochrome(s)
to the DNA of mammalian spermatozoa contained within previously frozen and then thawed
semen. In addition, the invention further involves devices, methods, and compositions for
15 the use of high purity separated X-chromosome bearing and Y-chromosome bearing
populations of spermatozoa from previously frozen-thawed semen in processes involving, but
not limited to, artificial insemination, surgical insemination, and in-vitro fertilization and
embryo culturing techniques.

20 II. BACKGROUND

Sperm can be collected from a great variety of mammals and then separated into X-
chromosome bearing and Y-chromosome bearing populations based upon the difference in
DNA content. In some conventional methods of spermatozoa separation, the DNA content of
the spermatozoa to be separated can be stained with a fluorochrome(s) that upon excitation
25 emit(s) a measurable amount of fluorescence. Because X-chromosome bearing spermatozoa
contain a greater amount of DNA than Y-chromosome bearing spermatozoa, each X-
chromosome bearing spermatozoa has the capacity to bind a relatively greater amount of
fluorochrome than the corresponding Y-chromosome bearing spermatozoa. Comparison of
the relative magnitude of emitted fluorescence upon excitation of the fluorochrome(s) allows
30 the isolation of X-chromosome bearing spermatozoa from Y-chromosome bearing
spermatozoa as described by United States Patent No. 5,135, 759, hereby incorporated by
reference.

Even though X-chromosome bearing spermatozoa and Y-chromosome bearing spermatozoa have been differentiated by and separated based upon the difference in emitted fluorescence for many years, and even though there is large commercial market for isolated populations of X-chromosome bearing spermatozoa and Y-chromosome bearing spermatozoa, there remain significant problems yet to be resolved.

A significant problem with conventional methods of separating X-chromosome bearing spermatozoa from Y-chromosome bearing spermatozoa can be that each resulting population contains a significant number of incorrectly separated spermatozoa that belong in the other population. This problem in differentiating between spermatozoa can, in part, be attributed to the lack of uniformity in the amount of fluorochrome bound to the spermatozoal DNA. As such, a range in the amount of fluorochrome bound by X-chromosome bearing spermatozoa is generated and a range in the amount of fluorochrome bound by Y-chromosome bearing spermatozoa is generated. When these ranges in the amount of fluorochrome overlap or yield some values that are similar, it can be difficult or impossible to classify those individual spermatozoa to one population or the other with any degree of certainty and cross contamination of the populations can occur.

This particular problem can be exacerbated with regard to spermatozoa obtained from frozen and subsequently thawed mammalian semen. The mean purity for separated Y-chromosome bearing spermatozoa population derived from previously frozen-thawed semen can be 85% or less, and the mean purity for separated X-chromosome bearing spermatozoa population derived from previously frozen-thawed semen can be 82% or less.

Another significant problem associated with staining of spermatozoal DNA can be the detrimental effects on fertilization rates and subsequent embryonic development of fertilized oocyte(s) (oocyte, ootid, or ovum, or a plurality of same, as may be appropriate within a specific application). One aspect of this problem may be that the amount of stain bound to the DNA may effect the viability of the spermatozoa resulting in lower fertilization rates. Another aspect of this problem can be that the amount of time that elapses during the staining of the DNA may effect the viability of the sperm resulting in lower fertilization rates.

Another aspect of this problem may be that the amount of time that elapses during staining of the DNA may lower subsequent cleavage rates of oocytes fertilized with such stained spermatozoa. A 20% decline in cleavage rates have been observed for oocytes when staining time requires 190 minutes as compared to when staining time requires 60 minutes. Another aspect of this problem may be that the percent of oocytes fertilized with stained spermatozoa that proceed to blastulation may be lower as described in the journal article entitled "In vitro Fertilization with Flow-Cytometrically-Sorted Bovine Sperm", Theriogenology 52: 1393-1405 (1999), hereby incorporated by reference herein.

Another significant problem may be that cryopreserved sperm may demonstrate increased capacitation, and the length of time such spermatozoa are viable may be shortened. As such, if previously frozen spermatozoa are to be separated into X-chromosome bearing and Y-chromosome bearing populations that are to be subsequently used in applications such as in-vitro fertilization, in-vivo artificial insemination, or the like, then routine staining procedures may have to be abbreviated to maintain suitable number of viable sperm cells.

As relating to the problems of staining spermatozoa uniformly, even when spermatozoa are obtained from previously frozen-thawed semen; maintaining sperm viability; separating stained spermatozoa into X-chromosome bearing and Y-chromosome bearing populations, even when the spermatozoa being separated are obtained from previously frozen semen; generating populations of X-chromosome bearing and Y-chromosome bearing spermatozoa having high purity; and successfully using separated spermatozoa for artificial insemination, surgical insemination, and in-vitro fertilization techniques it can be understood there are significant problems with conventional technology which are addressed by the instant invention.

III. DISCLOSURE OF THE INVENTION

A broad object of embodiments of the invention can be to provide DNA staining technology that allows substantially uniform amounts of fluorochrome to be bound to the DNA of all individual spermatozoa bearing an X-chromosome and substantially uniform

amounts of fluorochrome to be bound to all individual spermatozoa bearing a Y-chromosome within an amount of semen.

One aspect of this broad object of the invention can be to narrow the range in
5 magnitude of emitted fluorescence for each of the X-chromosome bearing population and the Y-chromosome bearing population of spermatozoa upon passing through a fluorochrome excitation source.

Another aspect of this broad object of the invention can be to increase the difference
10 between the mean values of magnitude of emitted fluorescence for each of the X-chromosome bearing population and the Y-chromosome bearing population of spermatozoa upon passing through a fluorochrome excitation source.

Another aspect of this broad object of the invention can be to decrease the number
15 of spermatozoa incorrectly assigned to each of the X-chromosome bearing population and the Y-chromosome bearing population of spermatozoa.

Another aspect of this broad object of the invention can be to generate separate X-chromosome bearing and Y-chromosome bearing populations having greater than 85%
20 purity or greater than 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or even 99% purity.

Another broad object of embodiment of the invention can be to allow assessment of a wide range of genetics. Rather than being limited to the genetics of individuals from species
25 of mammals having proximity to a spermatozoa separating or sorting facility, genetics representing a wide variety of individuals from numerous species can be transported as frozen semen to distant spermatozoa separation facilities for subsequent separation into X-chromosome bearing and into Y-chromosome bearing populations. These species of mammals may include, but are not limited to primates, such as chimpanzees, gorillas,
30 humans, or the like; marine mammals, such as whales, porpoises, or the like; bovids; ovids; swine; canids; felids; or equids, as but a few examples. It may also include genetics that are

considered rare because the species of mammal may be endangered or few in number; or considered rare because the individual has desirable morphological, physiological, or intellectual attributes.

5 Another broad object of embodiments of the invention can provide separation technology for differentiating between X-chromosome bearing and Y-chromosome bearing spermatozoa obtained from frozen-thawed semen.

10 Another object of embodiments of the invention can be to provide DNA staining technology to more uniformly stain the DNA of spermatozoa contained in frozen-thawed semen to improve the apparent resolution between X-chromosome bearing and Y-chromosome bearing spermatozoa.

15 Another object of embodiments of the invention can be to provide high purity artificial insemination samples prepared from separated spermatozoa from frozen-thawed semen.

20 Another object of embodiments of the invention can be to provide high purity low dose artificial insemination samples prepared from separated spermatozoa from frozen-thawed semen.

25 Another object of embodiments of the invention can be to provide high purity insemination samples for surgical insemination procedures prepared from separated spermatozoa from frozen-thawed semen.

 Another object of an embodiment of the invention can be to provide high purity insemination samples for in-vitro fertilization procedures prepared from separated spermatozoa from frozen-thawed semen.

30 Another object of an embodiment of the invention can be to provide high purity insemination samples for in-vitro fertilization procedures prepared from separated

spermatozoa from frozen-thawed human semen.

Another object of an embodiment of the invention can be to provide technology for staining and separation of spermatozoa from frozen-thawed sperm into X-chromosome bearing populations and Y-chromosome bearing populations for in-vitro fertilization of oocyte(s) that is not detrimental to cleavage rates or embryonic development.

Naturally further objects of the invention are disclosed throughout other areas of specification.

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IV. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a particular embodiment of the invention for staining the DNA of spermatozoa contained in frozen-thawed semen.

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Figure 2 shows a particular embodiment of the invention for separating spermatozoa from frozen-thawed semen into X-chromosome bearing and Y-chromosome bearing spermatozoa.

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Figure 3 shows a further view of a particular embodiment of the invention for separating spermatozoa from frozen-thawed semen into X-chromosome bearing and Y-chromosome bearing spermatozoa.

V. MODE(S) FOR CARRYING OUT THE INVENTION

To routinely separate spermatozoa (live, fixed, viable, non-viable, or nuclei) into high purity X-chromosome bearing samples and into Y-chromosome bearing samples, the method used to sort the X-chromosome bearing and Y-chromosome bearing spermatozoa must provide sufficient resolution of the X-chromosome bearing spermatozoa from the Y-chromosome bearing spermatozoa so that separation or sorting step(s) can be achieved without substantial cross contamination.

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Resolution or differentiation of spermatozoa can be based upon ascertaining the

difference in the fluorescent emission from the amount of fluorochrome bound to the DNA within the X-chromosome bearing spermatozoa upon excitation and the fluorescent emission from the amount of fluorochrome bound to the DNA within the Y-chromosome bearing spermatozoa upon excitation. Separation of X-chromosome bearing spermatozoa and Y-chromosome bearing spermatozoa based upon this measurable difference may then be achieved by a number of methods such as flow cytometry, liquid chromatography, gel electrophoresis, and other technologies that similarly compare the relative magnitude of fluorescence to differentiate between X-chromosome bearing spermatozoa and the Y-chromosome bearing spermatozoa.

Spermatozoa separation systems can have problems differentiating between the fluorescent emission generated by the fluorochrome bound to the DNA of X-spermatozoa, and the fluorescent emission generated by the fluorochrome bound to the DNA of Y-spermatozoa upon excitation when the amount of the fluorochrome bound to the DNA of individual spermatozoa is not consistent within the Y-chromosome bearing or X-chromosome bearing populations. These difficulties in differentiating between the amount of fluorescent emissions generated by the bound fluorochrome(s) become exacerbated when spermatozoa are obtained from frozen-thawed sperm which are stained by conventional techniques.

The failure to stain the spermatozoal DNA consistently can generate a broader range of fluorescing species for both X-chromosome bearing and Y-chromosome bearing populations of spermatozoa. This broader range of fluorescing species for the two populations results in an increased range of apparent DNA molecular weights and a decreased ability to resolve X-chromosome bearing from Y-chromosome bearing spermatozoa. The decrease in resolution makes separation of the X-chromosome bearing spermatozoa from the Y-chromosome bearing spermatozoa more difficult and results in cross contamination between populations and a lower purity of separated spermatozoa samples are obtained.

Particular embodiments of the invention provide technology to stain the DNA of live

5 viable spermatozoa or the spermatozoal DNA of frozen-thawed semen specimens to allow increased resolution of X-chromosome bearing from the Y-chromosome bearing spermatozoa resulting in high purity X-chromosome bearing and high purity Y-chromosome bearing populations of sperm cells. As such, it is understood that the term high purity can mean greater resolution of the X-chromosome bearing from the Y-chromosome bearing spermatozoa compared to conventional staining technology for a given application. High purity can also mean less cross contamination between separated spermatozoa populations compared to conventional separation technologies.

10 For example, in particular flow cytometry embodiments of the invention, high purity for stained frozen-thawed live spermatozoa can mean sorted populations of X-chromosome bearing spermatozoa and Y-chromosome bearing spermatozoa having a purity greater than about 85%. However, if live viable sperm or sperm nuclei are being sorted high purity may mean X-chromosome bearing and Y-chromosome bearing populations having a purity greater
15 than about 90%. As can be understood, the definition of high purity is contextual involving a comparison of the results obtained from each embodiment of the invention compared to the results obtained when utilizing convention technologies for a particular application. In the context of spermatozoa having DNA that stains poorly, such as previously frozen-thawed spermatozoal DNA, high purity can mean populations of isolated spermatozoa bearing
20 greater than 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of either an X-chromosome or a Y-chromosome.

Embodiments of the invention can include spermatozoa collected from numerous species of male mammals, and the invention should be understood not to be limited to the
25 species of male mammals described by the specific examples within this application. Rather the specific examples within this application are intended to be illustrative of the varied and numerous species of male mammals from which semen can be collected and utilized in certain embodiments of the invention. Embodiments of the invention, for example, may include the spermatozoa of animals having commercial value for meat or dairy production
30 such as swine, ovids, bovids, equids, buffalo, or the like (naturally the mammals used for meat or dairy production may vary from culture to culture). It may also include the

spermatozoa of various domesticated mammalian species encompassed by canids and felids. It may also include spermatozoa from individuals of various mammalian species that have uncommon attribute(s), such as morphological characteristics including weight, size, or conformation, or other desired characteristics such as speed, agility, intellect, or the like. It may also include spermatozoa of primates, including but not limited to chimpanzees, gorillas, or humans and the spermatozoa from marine mammals such as whales and dolphins. It may also include frozen-thawed spermatozoa from all the various mammals above-described and further, including but not limited to, the spermatozoa of deceased donors, from rare or exotic mammals, zoological specimens, or endangered species.

10

Now referring primarily to Figure 1, particular embodiments of the invention can comprise semen containing spermatozoa (1) collected from a male mammal, including but not limited to, those above-described. The spermatozoa can be incubated in a concentration of Hoechst 33342 stain (2) of greater than about 40 μM at a temperature between about 30 ° Centigrade and about 40° Centigrade for a duration of time between 50 minutes to 200 minutes to stain spermatozoal DNA with sufficient uniformity to allow X-chromosome bearing spermatozoa to be differentiated from Y-chromosome bearing spermatozoa based upon the magnitude of fluorescence at a rate greater than about 85%.

20

The concentration of Hoechst 33342 stain between 40 μM and 2500 μM , the temperature between 30 ° Centigrade and about 40° Centigrade, and the duration of time between 50 minutes and 200 minutes can be selected to adjust the purity of the separated X-chromosome bearing and Y-chromosome bearing populations, or can be selected to promote cleavage rates and embryonic development, as further discussed below.

25

For example, when staining spermatozoal DNA from certain bovine species, the concentration of Hoechst 33342 can be increased to between about 200 μM and about 2500 μM , incubated for a period of time between about 60 minutes to about 190 minutes at a temperature of about 37 ° Centigrade. Specifically with respect to certain frozen-thawed bovine spermatozoa, the Hoechst 33342 stain (2) can be adjusted to establish a concentration of 2240 μM and then incubated for about 60 minutes at about 39 ° Centigrade.

30

With respect to the cleavage rates of oocytes inseminated with mammalian sperm cells treated according to the invention, the increase in stain concentration up to at least 2240 μ M does not appear to have a depressive effect on either cleavage or embryonic development. Higher stain concentrations may actually be beneficial with respect to certain
5 embodiments of the invention because the length of incubation time may be decreased improving percent cleavage or blastocyst formation. From application to application the concentration of Hoechst 33342, the length of incubation time, or both can be adjusted to obtain the maximal cleavage rate and blastocyst formation, if desired.

10 Now referring primarily to Figures 2 and 3, flow cytometric embodiments of the invention can include a cell source (3) which acts to establish or supply stained spermatozoa (fresh, frozen-thawed, sperm nuclei, or the like) to be analyzed by flow cytometry. The cells are deposited within a nozzle (4) in a manner such that the stained sperm cells are surrounded by a sheath fluid (5). The sheath fluid (5) is usually supplied by a sheath fluid source (6) so
15 that as the cell source (3) supplies sperm cells, the sheath fluid (5) is concurrently fed through the nozzle (4). In this manner the sheath fluid (5) forms a sheath fluid environment for the sperm cells. Since the various fluids are provided to the flow cytometer at some pressure, they flow out of the nozzle (4) and exit at the nozzle orifice (7). By providing a type of oscillator (8) which may be very precisely controlled through an oscillator control (9),
20 pressure waves may be established within the nozzle (4) and transmitted to the fluids exiting the nozzle (4) at the nozzle orifice (7). Since the oscillator (9) acts upon the sheath fluid (5), the stream (10) exiting the nozzle orifice (7) eventually and regularly forms drops (11). Because the sperm cells are at least partially surrounded by a sheath fluid environment, the drops (11) can contain within them individually isolated sperm cells.

25

Since the drops (11) generally contain individual isolated sperm cells, the flow cytometer can distinguish and separate droplets based upon the magnitude of fluorescence emitted from the fluorochrome bound to the spermatozoal DNA. This is accomplished through a cell sensing system (12). The cell sensing system involves at least some type of
30 sensor (13) which responds to the magnitude of fluorescence emitted by each sperm cell contained within each drop (11). The sperm cell sensing system (13) may cause an action

depending upon the relative presence or relative absence of fluorescence emitted by the bound fluorochrome upon excitation by some stimulant such as the laser exciter (14). While each spermatozoon can be stained by the fluorochrome, such as Hoechst 33342, as described above, the differing amount of DNA comprising the X-chromosome and the Y-chromosome
5 causes different amounts of stain to be bound. Thus, by sensing the degree of fluorescence emitted by the fluorochrome upon excitation it is possible to discriminate between X-bearing spermatozoa and Y-bearing spermatozoa by their differing emission levels.

In order to achieve separation and isolation of the appropriate sperm cells, the signals
10 received by sensor (14) are fed to some type of sorter discrimination system (15) which very rapidly makes a differentiation decision and can differentially charge each drop (11) based upon whether it has decided that the desired sperm cell does or does not exist within that drop (11). In this manner the separation or discrimination system (15) acts to permit the electrostatic deflection plates (16) to deflect drops (11) based on whether or not they contain
15 the appropriate sperm cell. As a result, the flow cytometer acts to sort cells by causing them to land in one or more collectors or containment elements (17). Thus by sensing some property of the sperm cells (such as magnitude of fluorescence), the flow cytometer can discriminate between sperm cells based on that particular characteristic and place them in the appropriate collector or containment element (17). In particular embodiments of the
20 invention using flow cytometry to sort spermatozoa, the X-bearing sperm cell containing droplets are charged positively and thus deflect in one direction, and the Y-bearing sperm cell containing droplets are charged negatively and thus deflect the other way, and the wasted stream (containing unsortable sperm cells) remain uncharged and thus can be collected in an undeflected stream into a suction tube, or the like.

25

Now referring primarily to Figure 3, the nozzle (4) emits a stream (10) which because of the oscillator (8) (not shown in Figure 3) forms drops (11). Since the sperm cell source (3) (not shown in Figure 3) may supply sperm cells (1) which may be stained according to the above-described invention, the light emission from the bound fluorochrome excited by laser
30 exciter (13) can be differentially determined by sensor (14) so that the existence or nonexistence of a charge on each drop (11) as it separates from stream (10) can be controlled

by the flow cytometer. This control results in positively charged, negatively charged, or uncharged drops (8) based upon the sperm cell contained within each drop (11). As shown by Figure 3, certain drops are shown as deflected drops (18). These deflected drops (18) are those containing spermatozoon differentiated by bearing either an X-chromosome or a Y-chromosome. Separated spermatozoa are then isolated in an appropriate collection element or containment element (17) for later use.

Embodiments of the invention can comprise droplets (11) each containing a sperm cell (15) bearing either an X-chromosome or a Y-chromosome. Droplets containing X-chromosome bearing sperm cells can be isolated into containment element(s) (17) at a rate of at least 1000 per second or at a rate greater than about 1000 per second, such as 2000 per second, 3000 per second, 4000 per second, 5000 per second, or higher. Similarly Y-chromosome bearing sperm cells can be isolated at a rate of at least 1000 per second or at a rate greater than about 1000 per second, such as 2000 per second, 3000 per second, 4000 per second, 5000 per second, or higher. In some embodiments of the invention, droplets containing X-chromosome bearing sperm cells and droplets containing Y-chromosome bearing sperm cells are simultaneously separated and isolated into containment elements each at a rate of at least 1000 per second, or greater than 1000 per second, such as 2000 per second, 3000 per second, 4000 per second, 5000 per second, or at even higher rates.

Embodiments of the invention can also include artificial insemination samples prepared from sperm cells collected from male mammals (which can be frozen and thawed with respect to some embodiments of the invention) that are then stained and separated according to embodiments of the invention above-described. The artificial insemination samples can then be utilized in artificial insemination protocols. For example, a bovine artificial insemination sample prepared from separated spermatozoa according to the invention can comprise fewer than 10×10^6 viable spermatozoa contained within a straw. Low dose artificial insemination samples for bovine artificial insemination can contain as few as $1-3 \times 10^6$ viable spermatozoa, or even as few as 150,000 spermatozoa as described in United States Patent Application 09/001,394, or PCT Patent Application US98/27909, each hereby incorporated by reference. Artificial insemination samples, having a regular number

of separated sperm cells or a low dose of separated sperm cells can be used in animal breeding programs, such as those described in United States Patent Applications 60/224,050 and 60/21,093, each hereby incorporated by reference. Artificial insemination samples containing previously frozen and thawed spermatozoa stained and separated according to the invention can also be utilized in conjunction with synchronized breeding programs using superovulated animals as described in United States patent Application 09/001,454, hereby incorporated by reference herein. Naturally, for frozen sperm cells that are of limited availability because the male mammal is deceased, or the male mammal is a rare or exotic animal, an artificial insemination sample prepared according to the invention may contain even fewer spermatozoa.

The number of viable separated spermatozoa that are stained, separated, and isolated into X-chromosome bearing or Y-chromosome bearing populations according to the invention that are used in an artificial insemination sample can vary based upon the species of mammal to be artificially inseminated. For example, equine artificial insemination samples prepared from separated spermatozoa may require a higher number of viable separated spermatozoa relative to the bovine application, as described in PCT Patent Application US99/17165, hereby incorporated by reference. An embodiment of an equine insemination sample may, as but one example, contain between about forty million to about one-hundred million spermatozoa.

In certain embodiments of the invention, the insemination sample containing separated spermatozoa collected from a male mammal or obtained from frozen-thawed sperm may be packaged for use with surgical insemination procedures

Sperm cells stained, separated, or isolated according to the invention can also be used to fertilize oocyte(s) in-vitro (IVF). An attractive feature of IVF can be that fewer separated sperm are need than for artificial insemination. It may be desirable to use the fewest sperm possible, especially if the male mammal is deceased, rare, or exotic or if the spermatozoa are stained or separated in accordance with various embodiments of the invention. Also, commercial availability of sperm cells separated into X-chromosome bearing and Y-

chromosome bearing populations, especially when the male mammal is located a distance from the female mammal, or is exotic, rare, or has desirable attributes, will likely result in greatly expanded use of IVF in breeding programs. Certain embodiments of the invention can include devices and methodologies for the use of separated spermatozoa, including but not limited to frozen-thawed sperm cells, with respect to the in-vitro fertilization of oocytes, the in-vitro oocyte maturation, or the in-vitro culture of zygotes, such as those described in the journal article by Lu, K.H., Cran D.G., and Seidel, G. E., In-vitro Fertilization With Flow Cytometrically-Sorted Bovine Sperm, Theriogenology, 52, 1393-1405 (1999), hereby incorporated by reference.

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Certain embodiments of the invention involving the production or generation of mammalian embryos can comprise collection of semen (1) from a male mammal or obtaining semen or spermatozoa (1) that are or have been previously frozen. According to embodiments of the invention described above, the semen is combined with Hoechst 33342 (2) stain to establish a concentration of between 40 μM and 2500 μM . The sperm cells are incubated with the Hoechst 33342 stain at a temperature between about 30 ° Centigrade and about 40° Centigrade for a duration of between about 50 minutes to about 200 minutes. The stained sperm cells may be separated and isolated into X-chromosome bearing and Y-chromosome bearing populations according to embodiments of the invention described above or by other sperm cell separation techniques that also differentiate X-chromosome bearing spermatozoa from Y-chromosome bearing spermatozoa based upon the magnitude of fluorescence. The isolated sperm cells may then be used to fertilize oocytes from a female mammal of the same species, and in some cases from female mammals of different species, in-vitro.

25

As an example of an application of embodiments of the invention involving frozen bull sperm in IVF applications, sperm samples from two bulls were stained either at a concentration of 224 μM or 2,240 μM of Hoechst 33342 and the stained spermatozoa were then bulk sorted on a flow cytometer at 1000 sperm/sec into 2% egg yolk citrate. Spermatozoa were inseminated at $1 \times 10^6/\text{mL}$ and embryos were cultured in the mSOF system described by Tervit H.R. et al., Successful Culture In-Vitro of Sheep and Cattle Ova, J.

30

Reprod. Fertil., 30:493-497 (1992), hereby incorporated by reference. Three replicates were carried out for bull 1 and one replicate for bull 2 (Table 1).

Table 1. Effect of stain concentration on cleavage and developmental rates of oocytes inseminated with separated stained spermatozoa from frozen-thawed sperm.

Bull	No. Ejaculates	Hoechst 33342 conc. (µM)	Staining time required (min)	No. oocytes	% cleave	% blastocysts/oocyte
1	3	224	190	368	44 ^a	17
1	3	2240	60	373	60 ^b	23
2	1	224	190	86	23 ^a	0 ^a
2	1	2240	60	81	42 ^b	16 ^b

^{a,b} Percentages within bulls within columns with different superscripts differ ($P<0.025, \chi^2$)

As can be understood , It can take much longer to stain frozen-thawed sperm so that they can be resolved during separation at the lower stain concentration than at 10X stain concentration. The differences observed in cleavage rates between the two stain concentrations most likely can be attributed to the extended incubation time at the lower stain level. It appears that a 10-fold increase in stain concentration does not have depressive effect on either cleavage of embryonic development.

As can be easily understood from the foregoing, the basic concepts of the present invention may be embodied in a variety of ways. It involves the staining of spermatozoa, whether fresh spermatozoa or frozen-thawed spermatozoa, separation and isolation techniques which may be used with such stained spermatozoa, as well as devices to accomplish the staining, separation, and isolation of such stained spermatozoa into X-chromosome bearing and Y-chromosome bearing populations. In this patent application, the staining and separating techniques used with spermatozoa are disclosed as part of the results

shown to be achieved by the various devices described and as steps which are inherent to utilization. They are simply the natural result of utilizing the devices as intended and described. In addition, while some devices are disclosed, it should be understood that these not only accomplish certain methods but also can be varied in a number of ways.

5 Importantly, as to all of the foregoing, all of these facets should be understood to be encompassed by this disclosure.

The discussion included in this international Patent Cooperation Treaty patent application is intended to serve as a basic description. The reader should be aware that the

10 specific discussion may not explicitly describe all embodiments possible; many alternatives are implicit. It also may not fully explain the generic nature of the invention and may not explicitly show how each feature or element can actually be representative of a broader function or of a great variety of alternative or equivalent elements. Again, these are implicitly included in this disclosure. Where the invention is described in functionally-

15 oriented terminology, each aspect of the function is accomplished by a device, subroutine, or program. Apparatus claims may not only be included for the devices described, but also method or process claims may be included to address the functions the invention and each element performs. Neither the description nor the terminology is intended to limit the scope of the claims which now be included.

20

Further, each of the various elements of the invention and claims may also be achieved in a variety of manners. This disclosure should be understood to encompass each such variation, be it a variation of an embodiment of any apparatus embodiment, a method or process embodiment, or even merely a variation of any element of these. Particularly, it

25 should be understood that as the disclosure relates to elements of the invention, the words for each element may be expressed by equivalent apparatus terms or method terms -- even if only the function or result is the same. Such equivalent, broader, or even more generic terms should be considered to be encompassed in the description of each element or action. Such terms can be substituted where desired to make explicit the implicitly broad coverage to

30 which this invention is entitled. As but one example, it should be understood that all actions may be expressed as a means for taking that action or as an element which causes that action.

Similarly, each physical element disclosed should be understood to encompass a disclosure of the action which that physical element facilitates. Regarding this last aspect, as but one example, the disclosure of a "sorter" should be understood to encompass disclosure of the act of "sorting" -- whether explicitly discussed or not -- and, conversely, were there only
 5 disclosure of the act of "sorting", such a disclosure should be understood to encompass disclosure of a "sorter" and even a "means for sorting". Such changes and alternative terms are to be understood to be explicitly included in the description. Additionally, the various combinations and permutations of all elements or applications can be created and presented. All can be done to optimize the design or performance in a specific application.

10

Any acts of law, statutes, regulations, or rules mentioned in this application for patent: or patents, publications, or other references mentioned in this application for patent are hereby incorporated by reference. Specifically, United States Provisional Patent Application No. 60/253,787, filed November 29, 2000 and United States Provisional Patent Application
 15 No. 60/253,785, filed November 29, 2000, are hereby incorporated by reference including any figures or attachments, and each of references in the following table of references are hereby incorporated by reference.

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US Application 60/211093, entitled "Integrated System for Herd Management Using Sexed Semen", filed June 12, 2000.
US Application entitled "System For Separating Frozen-Thawed Sperm Cells Into X-Chromosome And Y-Chromosome Bearing Populations", filed November 28, 2000.
US Application Serial Number 60/094,720, entitled "System for Low Dose Insemination of Equines", filed July 30, 1998.
US Application Serial Number 60/113,143, entitled "Equine Insemination System", December 18, 1998.
US Application Serial Number 60/203,089, entitled "Detector System for Resolving Small Differences in Photo-generated Signal", filed May 9, 2000.
US Application Serial Number 60/211093, entitled "Integrated System for Herd Management Using Sexed Semen", filed June 12, 2000.
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In addition, as to each term used it should be understood that unless its utilization in this application is inconsistent with such interpretation, common dictionary definitions should be understood as incorporated for each term and all definitions, alternative terms, and synonyms such as contained in the Random House Webster's Unabridged Dictionary, second edition are hereby incorporated by reference. However, as to each of the above, to the extent that such information or statements incorporated by reference might be considered inconsistent with the patenting of this/these invention(s) such statements are expressly not to be considered as made by the applicant(s).

In addition, unless the context requires otherwise, it should be understood that the term "comprise" or variations such as "comprises" or "comprising", are intended to imply the inclusion of a stated element or step or group of elements or steps but not the exclusion of any other element or step or group of elements or steps. Such terms should be interpreted in their most expansive form so as to afford the applicant the broadest coverage legally permissible in countries such as Australia and the like.

Thus, the applicant(s) should be understood to have support to claim at least: i) each of the staining, separation, isolation, insemination, or fertilization procedures as herein disclosed and described, ii) the related methods disclosed and described, iii) similar, equivalent, and even implicit variations of each of these devices and methods, iv) those

alternative designs which accomplish each of the functions shown as are disclosed and described, v) those alternative designs and methods which accomplish each of the functions shown as are implicit to accomplish that which is disclosed and described, vi) each feature, component, and step shown as separate and independent inventions, vii) the applications
5 enhanced by the various systems or components disclosed, viii) the resulting products produced by such systems or components, ix) methods and apparatuses substantially as described hereinbefore and with reference to any of the accompanying examples, and x) the various combinations and permutations of each of the elements disclosed.

10 The claims set forth in this specification are hereby incorporated by reference as part of this description of the invention, and the applicant expressly reserves the right to use all of or a portion of such incorporated content of such claims as additional description to support any of or all of the claims or any element or component thereof, and the applicant further expressly reserves the right to move any portion of or all of the incorporated content of such
15 claims or any element or component thereof from the description into the claims or vice-versa as necessary to define the subject matter for which protection is sought by this application or by any subsequent continuation, division, or continuation-in-part application thereof, or to obtain any benefit of, reduction in fees pursuant to, or to comply with the patent laws, rules, or regulations of any country or treaty, and such content incorporated by
20 reference shall survive during the entire pendency of this application including any subsequent continuation, division, or continuation-in-part application thereof or any reissue or extension thereon.

VI CLAIMS

I claim:

- 5 1. A method of staining sperm cells collected from mammals, comprising the steps of:
- a. collecting semen from a male mammal;
 - b. incubating sperm cells contained within said semen in a concentration of Hoechst 33342 stain of greater than 40 micro-molar;
 - c. establishing the temperature at which said sperm cells in said concentration of
10 Hoechst 33342 stain are incubated between about 30 degrees centigrade and about 40 degrees centigrade;
 - d. adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes; and
 - 15 e. staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85%.
- 20 2. A method of staining sperm cells collected from mammals as described in claim 1, wherein said male mammal is selected from the group of mammals consisting of primates, humans, swine, ovids, bovids, equids, canids, felids, and dolphins.
3. A method of staining sperm cells collected from mammals as described in claim 2,
25 wherein said male mammal comprises said bovid and said concentration of Hoechst 33342 stain is between about 200 micro-molar and about 2500 micro-molar.
4. A method of staining sperm cells collected from mammals as described in claim 2,
30 wherein said male mammal comprises said bovid and said concentration of Hoechst 33342 stain is 224 micro-molar.

5. A method of staining sperm cells collected from mammals as described in claim 2, wherein said male mammal comprises said bovid and said concentration of Hoechst 33342 stain is 2240 micro-molar.
- 5
6. A method of staining sperm cells collected from mammals as described in claim 4, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 190 minutes.
- 10
7. A method of staining sperm cells collected from mammals as described in claim 5, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 60 minutes.
- 15
8. A method of staining sperm cells collected from mammals as described in claim 1, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said Y-chromosome bearing sperm cells from said X-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate selected from the group consisting of 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.
- 20
- 25
9. A method of staining sperm cells collected from mammals as described in claim 8, wherein said X-chromosome bearing sperm cells differentiated from said Y-chromosome bearing sperm cells comprise viable sperm cells.
- 30
10. A method of staining sperm cells collected from mammals as described in claims 1,

2, 6, 7, 8, or 9, further comprising the step of freezing said semen.

11. A method of staining sperm cells collected from mammals as described in claim 10, further comprising the step of thawing said semen.

5

12. A method of staining sperm cells collected from mammals as described in claim 11, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said magnitude of fluorescence with a flow cytometer.

10

13. A method of staining DNA within frozen-thawed sperm cells, comprising the steps of:

15

- a. collecting semen containing sperm cells from a male mammal;
- b. freezing said semen containing said sperm cells;
- c. thawing said semen containing said sperm cells;
- d. combining frozen-thawed semen with Hoechst 33342 stain;
- e. establishing a concentration of said Hoechst 33342 stain between about 200 micro-molar and about 2500 micro-molar;
- f. adjusting the temperature of said frozen-thawed semen in said concentration of Hoechst 33342 stain to between about 30 degrees Centigrade and about 40 degrees Centigrade; and
- g. adjusting the duration of time said frozen-thawed semen in said concentration of Hoechst 33342 stain incubates to between about 50 minutes and 200 minutes, whereby DNA within sperm cells contained in said frozen-thawed are stained with sufficient uniformity to differentiate between X-chromosome bearing sperm cells and Y-chromosome bearing sperm cells on the basis of magnitude of fluorescence.

20

25

30

14. A method of staining sperm cells collected from mammals as described in claim 13,

wherein said male mammal is selected from the group of mammals consisting of primates, humans, swine, ovida, bovida, equids, canids, felids, and dolphins.

15. A method of staining sperm cells collected from mammals as described in claim 14,
5 wherein said male mammal comprises said bovid and said concentration of Hoechst 33342 stain is between about 200 micro-molar and about 2500 micro-molar.
16. A method of staining sperm cells collected from mammals as described in claim 14,
10 wherein said male mammal comprises said bovid and said concentration of Hoechst 33342 stain is 224 micro-molar.
17. A method of staining sperm cells collected from mammals as described in claim 14,
15 wherein said male mammal comprises said bovid and said concentration of Hoechst 3342 stain is 2240 micro-molar.
18. A method of staining sperm cells collected from mammals as described in claim 14,
20 wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 190 minutes.
19. A method of staining sperm cells collected from mammals as described in claim 17,
25 wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 60 minutes.
20. A method of staining sperm cells collected from mammals as described in claims 13,
30 15, 16, 17, 18, 19, or 20, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of

fluorescence at a rate of greater than about 85% comprises differentiating said Y-chromosome bearing sperm cells from said X-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate selected from the group consisting of 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

5

21. A method of staining sperm cells collected from mammals as described in claim 20, wherein said X-chromosome bearing sperm cells differentiated from said Y-chromosome bearing sperm cells comprise viable sperm cells.

10 22. A method of staining sperm cells collected from mammals as described in claim 20, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said magnitude of fluorescence with a flow
15 cytometer.

23. A method of generating mammalian embryos, comprising the steps of:

- a. collecting semen from a male mammal;
- 20 b. combining said semen from said male mammal with an amount of Hoechst 33342 stain;
- c. establishing a concentration of Hoechst 33342 stain combined with said semen to a concentration between about 40 micro-molar and about 2500 micro-molar;
- d. adjusting the temperature at which said sperm cells are incubated with said
25 Hoechst 33342 stain between about 30 degrees centigrade and about 40 degrees centigrade;
- e. adjusting the duration of time said sperm cells are incubated in said concentration of Hoechst 33342 between about 60 minutes and about 200 minutes;
- 30 f. staining DNA within sperm cells contained in said semen with said Hoechst 33342 stain; and

- g. fertilizing oocytes with stained sperm cells,
whereby increasing the concentration of Hoechst 33342 stain and decreasing
the duration of time said sperm cells are incubated in said concentration of
Hoechst 33342 stain increases the percentage of said mammalian embryos
5 produced.
24. A method of staining sperm cells collected from mammals as described in claim 23,
wherein said male mammal is selected from the group of mammals consisting of
primates, humans, swine, ovids, bovids, equids, camids, felids, and dolphins.
10
25. A method of staining sperm cells collected from mammals as described in claim 24,
wherein said male mammal comprises said bovid and said concentration of Hoechst
33342 stain is between about 200 micro-molar and about 2500 micro-molar.
- 15 26. A method of staining sperm cells collected from mammals as described in claim 24,
wherein said male mammal comprises said bovid and said concentration of Hoechst
33342 stain is 224 micro-molar.
27. A method of staining sperm cells collected from mammals as described in claim 24,
20 wherein said male mammal comprises said bovid and said concentration of Hoechst
3342 stain is 2240 micro-molar.
28. A method of staining sperm cells collected from mammals as described in claim 26,
wherein said step of adjusting a duration of time said sperm cells are incubated with
25 said concentration of Hoechst 33342 stain between about 50 minutes and about 200
minutes comprises adjusting said duration of time said sperm cells are incubated with
said concentration of Hoechst 33342 stain to about 190 minutes.
29. A method of staining sperm cells collected from mammals as described in claim 27,
30 wherein said step of adjusting a duration of time said sperm cells are incubated with
said concentration of Hoechst 33342 stain between about 50 minutes and about 200

minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 60 minutes.

- 5 30. A method of staining sperm cells collected from mammals as described in claims 23, 25, 26, 27, 28, 29, or 30, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said Y-chromosome bearing sperm cells from said X-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate selected from the group consisting of 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.
- 10 31. A method of staining sperm cells collected from mammals as described in claim 30, wherein said X-chromosome bearing sperm cells differentiated from said Y-chromosome bearing sperm cells comprise viable sperm cells.
- 15 32. A method of staining sperm cells collected from mammals as described in claim 30, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosomes bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said magnitude of fluorescence with a flow cytometer.
- 20 33. A method of staining sperm cells collected from mammals as described in claim 32, further comprising the step of isolating differentiated X-chromosome bearing sperm cells and Y-chromosome bearing sperm cells into separate collection elements.
- 25 34. A method of staining sperm cells collected from mammals as described in claim 33, wherein said step of isolating differentiated X-chromosome bearing sperm cells and Y-chromosome bearing sperm cells into separate collection elements comprises isolating Y-chromosome bearing sperm cells into a separate collection element at a
- 30

rate of about 1000 per second.

35. A method of staining sperm cells collected from mammals as described in claim 33, wherein said step of isolating differentiated X-chromosome bearing sperm cells and
5 Y-chromosome bearing sperm cells into separate collection elements comprises isolating X-chromosome bearing sperm cells into a separate collection element at a rate of about 1000 per second.
36. A method of generating mammalian embryos as described in claims 23, further
10 comprising the step of freezing said semen.
37. A method of generating mammalian embryos as described in claims 30, further comprising the step of freezing said semen.
- 15 38. A method of generating mammalian embryos as described in claim 36, further comprising the step of thawing said semen.
39. A method of generating mammalian embryos as described in claim 37, further comprising the step of thawing said semen.
20
40. A flow cytometer system for isolating desired sperm cells, comprising:
a. sperm cells obtained by thawing previously frozen semen, wherein said sperm cells are incubated with a concentration of Hoechst 33342 stain between about 200 micro-molar and about 2500 micro-molar until DNA within said
25 sperm cells are stained with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85%;
- b. a sperm cell source that supplies said sperm cells to a flow cytometer;
- 30 c. a sheath fluid source that creates a sheath fluid environment within said flow cytometer in which said sperm cells are entrained;

- d. a nozzle through which said sperm cells pass while entrained in said sheath fluid environment;
 - e. an oscillator that acts upon said sheath fluid as it passes through said nozzle;
 - f. a sperm cell sensing system responsive to said sperm cells;
 - 5 g. a separation discrimination system that acts to separate said sperms cells having a desired characteristic; and
 - h. a containment element into which said sperm cells having said desired characteristic are collected.
- 10 41. A flow cytometer system for isolating desired sperm cells as described in claim 40, wherein said sperm cells obtained by thawing previously frozen semen are obtained from male mammals selected from the group consisting of primates, humans, swine, ovids, bovids, equids, canids, felids, and dolphins.
- 15 42. A flow cytometer system for isolating desired sperm cells as described in claim 41, wherein said male mammal comprises said bovid, and wherein said concentration of Hoechst 33342 stain is between about 200 micro-molar and about 2500 micro-molar.
- 20 43. A flow cytometer system for isolating desired sperm cells as described in claim 41, wherein said male mammal comprises said bovid, and wherein said concentration of Hoechst 33342 stain is about 224 micro-molar.
- 25 44. A flow cytometer system for isolating desired sperm cells as described in claim 41, wherein said male mammal comprises said bovid, and wherein said concentration of Hoechst 3342 stain is 2240 micro-molar.
- 30 45. A flow cytometer system for isolating desired sperm cells as described in claim 42, further comprising a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes.

46. A flow cytometer system for isolating desired sperm cells as described in claim 43, wherein said duration of time about 190 minutes.
47. A flow cytometer system for isolating desired sperm cells as described in claim 44,
5 wherein said duration of time about 60 minutes.
48. A flow cytometer system for isolating desired sperm cells as described in claims 40,
42, 43, 45, or 48, wherein said X-chromosome bearing sperm cells to be
differentiated from Y-chromosome bearing sperm cells based upon the magnitude of
10 fluorescence at a rate of greater than about 85% is a rate selected from the group
consisting of 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%,
98%, or 99%.
49. A flow cytometer system for isolating desired sperm cells as described in claim 48,
15 further comprising a collection element into which differentiated X-chromosome
bearing sperm cells are isolated.
50. A flow cytometer system for isolating desired sperm cells as described in claim 48,
further comprising a collection element into which differentiated Y-chromosome
20 bearing sperm are isolated.
51. A flow cytometer system for isolating desired sperm cells as described in claim 49,
further comprising a rate at which X-chromosome bearing sperm cells are isolated
greater than about 1000 per second.
25
52. A flow cytometer system for isolating desired sperm cells as described in claim 50,
further comprising a rate at which Y-chromosome bearing sperm cells are isolated
greater than about 1000 per second.
- 30 53. A method of generating mammalian embryos as described in claim 40, further
comprising the step of freezing said semen.

54. A method of generating mammalian embryos as described in claims 51, further comprising the step of freezing said semen.
- 5 55. A method of generating mammalian embryos as described in claim 53, further comprising the step of thawing said semen.
56. A method of generating mammalian embryos as described in claim 54, further comprising the step of thawing said semen.
- 10 57. A method of producing a mammal having a predetermined sex comprising the steps of:
- a. collecting semen from a male mammal;
 - b. freezing said semen;
 - 15 c. thawing said semen;
 - b. determining the sex characteristic of a plurality of sperm cells contained within said frozen-thawed semen;
 - c. separating said sperm cells according to the determination of their sex characteristic;
 - 20 d. isolating sperm cells separated according to the determination of their sex in a collection element;
 - d. establishing an artificial insemination sample from said sperm cells isolated in said collection element;
 - e. inserting said artificial insemination sample into a female mammal of the
 - 25 same species from which said semen was collected;
 - f. fertilizing at least one egg within said female mammal; and
 - g. producing an offspring mammal of the desired sex.
58. A method of producing a mammal having a predetermined sex as described in claim
- 30 57, wherein said male mammal is selected from the group of mammals consisting of primates, humans, swine, ovids, bovids, equids, canids, felids, and dolphins.

59. A method of producing a mammal having a predetermined sex as described in claim 58, further comprising the step of staining DNA within said sperm cells with a concentration of Hoechst 33342 greater than 40 micro-molar.
- 5
60. A method of producing a mammal having a predetermined sex as described in claim 59, wherein said step of staining DNA within said sperm cells with a concentration of Hoechst 33342 greater than 40 micro-molar comprises staining of sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85%.
- 10
61. A method of producing a mammal having a predetermined sex as described in claim 59, wherein said male mammal comprises said bovid, and wherein said concentration of Hoechst 33342 stain is between about 200 micro-molar and about 2500 micro-molar.
- 15
62. A method of producing a mammal having a predetermined sex as described in claim 61, wherein said male mammal comprises said bovid, and wherein said concentration of Hoechst 33342 stain is 224 micro-molar.
- 20
63. A method of producing a mammal having a predetermined sex as described in claim 61, wherein said male mammal comprises said bovid and wherein said concentration of Hoechst 3342 stain is 2240 micro-molar.
- 25
64. A method of producing a mammal having a predetermined sex as described in claim 61, further comprising the step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes.
- 30
65. A method of producing a mammal having a predetermined sex as described in claim

62, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 190 minutes.

5

66. A method of producing a mammal having a predetermined sex as described in claim 63, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 60 minutes.

10

67. A method of producing a mammal having a predetermined sex as described in claims 57, 58, 61, 62, 63, 64, 65, or 66, wherein said step of staining DNA within said sperm cells with a concentration of Hoechst 33342 greater than 40 micro-molar comprises staining of sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises a rate selected from the group consisting of 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

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68. A method of producing a mammal having a predetermined sex as described in claim 67, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said magnitude of fluorescence with a flow cytometer.

25

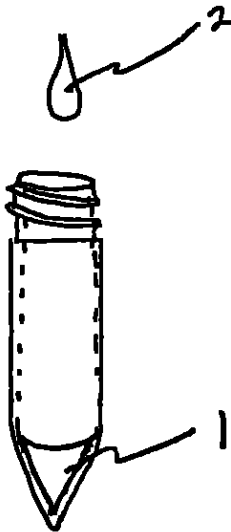
69. A method of staining sperm cells collected from mammals as described in claim 68, wherein said step of isolating sperm cells separated according to the determination of their sex in a collection element comprises isolating Y-chromosome bearing sperm cells into a separate collection element at a rate of about 1000 per second.

30

70. A method of staining sperm cells collected from mammals as described in claim 68, wherein said step of isolating sperm cells separated according to the determination of their sex in a collection element comprises isolating X-chromosome bearing sperm cells into a separate collection element at a rate of about 1000 per second.
- 5
71. A method of producing a mammal having a predetermined sex as described in claim 57, further comprising the step of limiting the number of isolated sperm cells in said artificial insemination sample to about 10% to about 50% of the number of said sperm cells relative to a typical unseparated artificial insemination sample.
- 10
72. A method of producing a mammal having a predetermined sex as described in claim 58, wherein said mammal comprises said bovid and wherein said artificial insemination sample has the number of isolated sperm cells limited to about one million to three million.
- 15
73. A method of producing a mammal having a predetermined sex as described in claim 58, wherein said mammal is a bovid and wherein said artificial insemination sample has the number of isolated sperm cells limited to between about one-hundred and fifty thousand and about one million.
- 20
74. A method of producing a mammal having a predetermined sex as described in claim 57, wherein said mammal comprises said equid and wherein said artificial insemination sample has the number of isolated sperm cells limited to between about forty million and about one hundred million.
- 25
75. A method of producing a mammal having a predetermined sex as described in claims 71, 72, 73 or 74, further comprising the step of creating superovulation in said female mammal to create at least two eggs comprising the step of using an ovulatory pharmaceutical to cause multiple eggs to be produced, and wherein said ovulatory pharmaceutical is injected in half day increments between any of days 2 and 18 of
- 30

the estrus cycle.

FIG 1



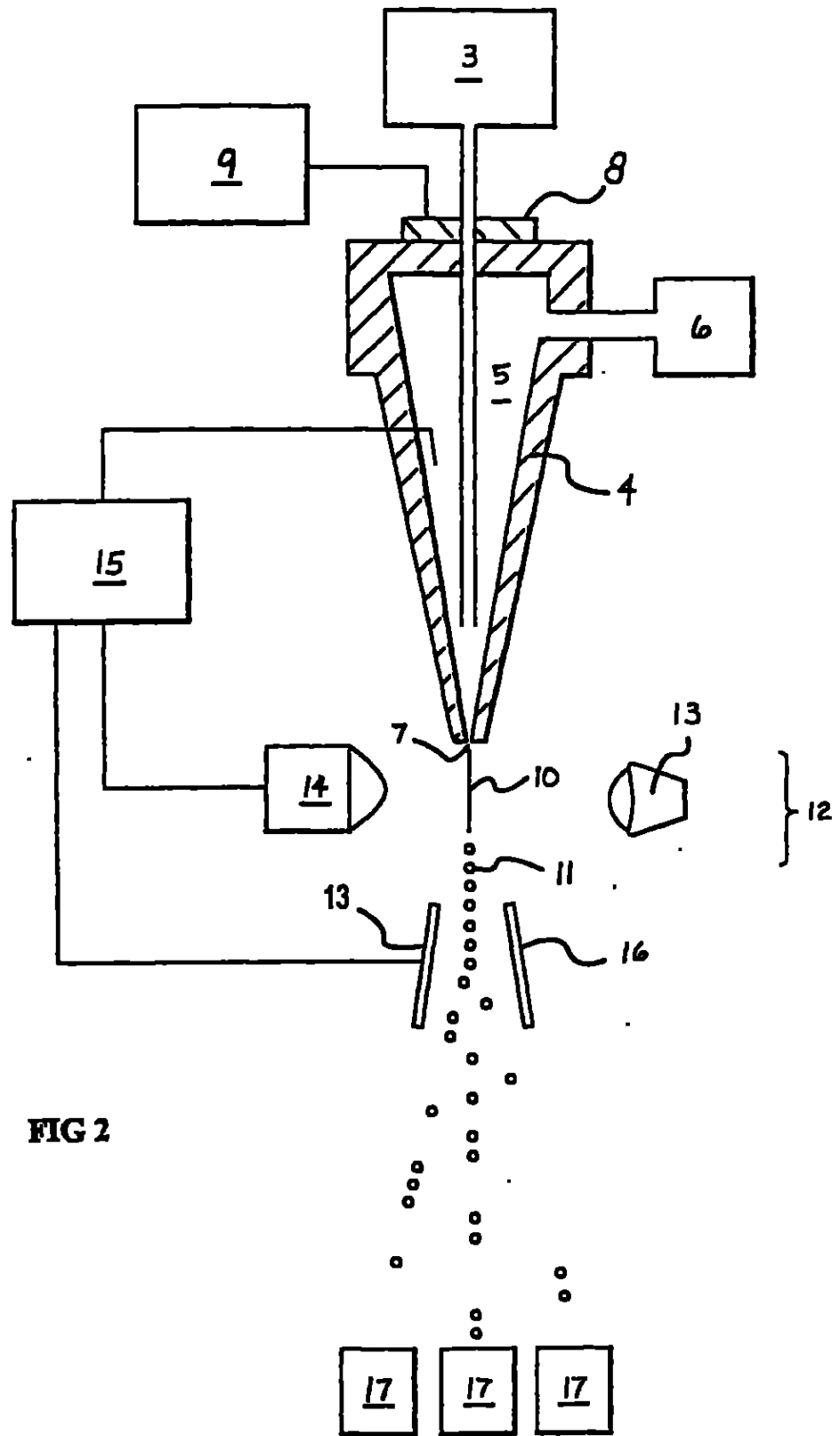


FIG 2

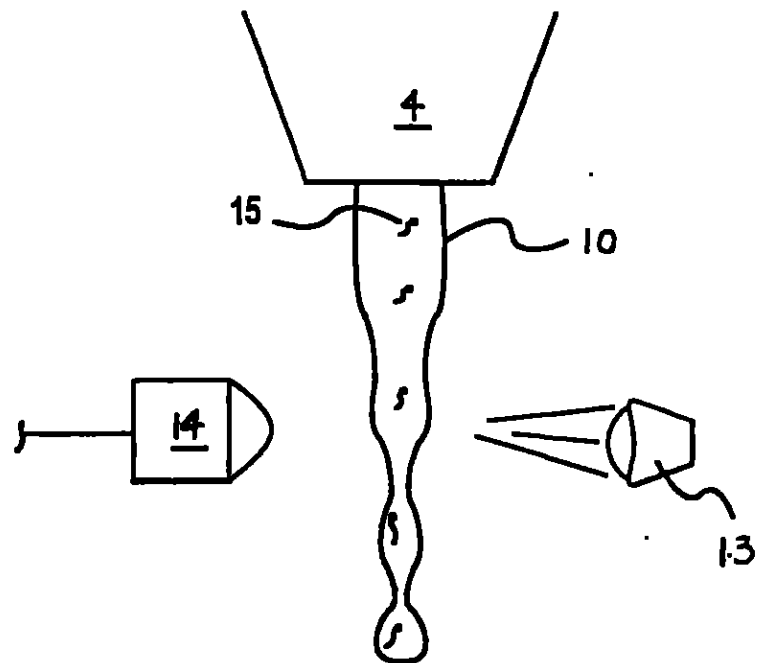
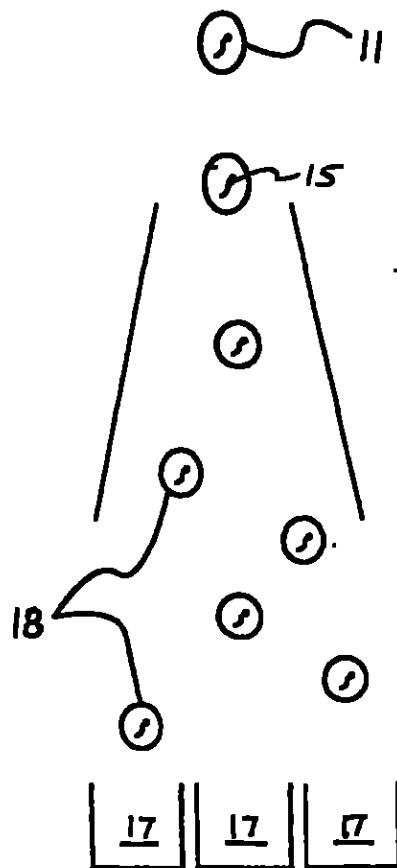


FIG 3



PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference
(if desired) (12 characters maximum) XYFrozSorPCT

Box No. I TITLE OF INVENTION System To Separate Frozen-Thawed Spermatozoa Into X-Chromosome Bearing And Y-Chromosome Bearing Populations	
Box No. II APPLICANT ☐ This person is also inventor	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) XY, INC. 1108 North Lemay Avenue Fort Collins, Colorado 80524 United States of America	
Telephone No. (970) 493-3113	
Facsimile No. (970) 493-3114	
Teleprinter No.	
Applicant's registration No. with the Office	
State (that is, country) of nationality: US	State (that is, country) of residence: US
This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box	
Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) COLORADO STATE UNIVERSITY through its agent Colorado State University Research Foundation 601 South Howes, Room 410 Fort Collins, Colorado 80522 United States of America	
This person is: ☒ applicant only ☐ applicant and inventor ☐ inventor only (If this check-box is marked, do not fill in below.)	
Applicant's registration No. with the Office	
State (that is, country) of nationality: US	State (that is, country) of residence: US
This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box	
☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.	
Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE	
The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) MILES, Craig R. Santangelo Law Offices, p.C. 125 South Howes, Third Floor Fort Collins, Colorado 80521 United States of America	
Telephone No. (970) 224-3100	
Facsimile No. (970) 224-3175	
Teleprinter No.	
Agent's registration No. with the Office 45,954	
☐ Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.	

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S) <i>If none of the following sub-boxes is used, this sheet should not be included in the request.</i>	
Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i> SEIDEL, George E. c/o XY, Inc. 1108 North Lemay Avenue Fort Collins, Colorado 80524 United States of America	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only <i>(If this check-box is marked, do not fill in below.)</i> Applicant's registration No. with the Office
State (that is, country) of nationality: US	State (that is, country) of residence: US
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i> SUH, Tae Kwang c/o XY, Inc. 1108 North Lemay Avenue Fort Collins, Colorado 80524 United States of America	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only <i>(If this check-box is marked, do not fill in below.)</i> Applicant's registration No. with the Office
State (that is, country) of nationality: KR	State (that is, country) of residence: US
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i> LU, Kehaun c/o XY, Inc. 1108 North Lemay Avenue Fort Collins, Colorado 80524 United States of America	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only <i>(If this check-box is marked, do not fill in below.)</i> Applicant's registration No. with the Office
State (that is, country) of nationality: UK	State (that is, country) of residence: UK
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box	
Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i> 	This person is: ☐ applicant only ☐ applicant and inventor ☐ inventor only <i>(If this check-box is marked, do not fill in below.)</i> Applicant's registration No. with the Office
State (that is, country) of nationality:	State (that is, country) of residence:
This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box	
☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.	

Box No. V DESIGNATION OF STATES *Mark the applicable check-boxes below: at least one must be marked.*

The following designations are hereby made under Rule 4.9(a):

Regional Patent

- ☒ AP ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, MZ Mozambique, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- (5) ☒ EA Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- (2) ☒ EP European Patent: AT Austria, BE Belgium, CH & LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, TR Turkey, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ OA OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT (If other kind of protection or treatment desired, specify on dotted line)

National Patent (If other kind of protection or treatment desired, specify on dotted line):

- | | | |
|---|--|---|
| ☒ AE United Arab Emirates | ☒ GH Ghana | (4) ☒ MX Mexico |
| ☒ AG Antigua and Barbuda | ☒ GM Gambia | ☒ MZ Mozambique |
| ☒ AL Albania | ☒ HR Croatia | ☒ NO Norway |
| ☒ AM Armenia | ☒ HU Hungary | ☒ NZ New Zealand |
| ☒ AT Austria and Utility Model | ☒ ID Indonesia | ☒ PL Poland |
| (5) ☒ AU Australia | ☒ IL Israel | ☒ PT Portugal |
| ☒ AZ Azerbaijan | ☒ IN India | ☒ RO Romania |
| ☒ BA Bosnia and Herzegovina | ☒ IS Iceland | ☒ RU Russian Federation |
| ☒ BB Barbados | ☒ JP Japan | |
| ☒ BG Bulgaria | ☒ KE Kenya | ☒ SD Sudan |
| ☒ BR Brazil | ☒ KG Kyrgyzstan | ☒ SE Sweden |
| ☒ BY Belarus | ☒ KP Democratic People's Republic of Korea | ☒ SG Singapore |
| ☒ BZ Belize | ☒ KR Republic of Korea | ☒ SI Slovenia |
| (3) ☒ CA Canada | ☒ KZ Kazakhstan | ☒ SK Slovakia & Utility Model |
| ☒ CH & LI Switzerland and Liechtenstein | ☒ LC Saint Lucia | ☒ SL Sierra Leone |
| ☒ CN China | ☒ LK Sri Lanka | ☒ TJ Tajikistan |
| ☒ CO Colombia | ☒ LR Liberia | ☒ TM Turkmenistan |
| ☒ CR Costa Rica | ☒ LS Lesotho | ☒ TR Turkey |
| ☒ CU Cuba | ☒ LT Lithuania | ☒ TT Trinidad and Tobago |
| ☒ CZ Czech Republic & Utility Model | ☒ LU Luxembourg | ☒ TZ United Republic of Tanzania |
| ☒ DE Germany & Utility Model | ☒ LV Latvia | ☒ UA Ukraine |
| ☒ DK Denmark & Utility Model | ☒ MA Morocco | ☒ UG Uganda |
| ☒ DM Dominica | ☒ MD Republic of Moldova | (1) ☒ US United States of America |
| ☒ DZ Algeria | | |
| ☒ EC Ecuador | | |
| ☒ EE Estonia and Utility Model | ☒ MG Madagascar | ☒ UZ Uzbekistan |
| ☒ ES Spain | ☒ MK The former Yugoslav Republic of Macedonia | ☒ VN Viet Nam |
| ☒ FI Finland and Utility Model | ☒ MN Mongolia | ☒ YU Yugoslavia |
| ☒ GB United Kingdom | ☒ MW Malawi | ☒ ZA South Africa |
| ☒ GD Grenada | | ☒ ZW Zimbabwe |
| ☒ GE Georgia | | |

Check-boxes below reserved for designating States which have become party to the PCT after issuance of this sheet

- ☒ All possible states & all ... ☒ GQ Equatorial Guinea ☒ PH Philippines
- ☒ possible countries that have become members of the PCT since issuance of this form ☒ OM Oman ☒ ZM Zambia

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. (Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)

Supplemental Box If the Supplemental Box is not used, this sheet should not be included in the request.

1. If, in any of the Boxes, except Boxes Nos. VII(f) to (v) for which a special continuation box is provided, the space is insufficient to furnish all the information: in such case, write "Continuation of Box No. ..." (indicate the number of the Box) and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:
 - (i) if more than two persons are to be indicated as applicants and/or inventors and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;
 - (ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be); indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;
 - (iii) if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be); indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;
 - (iv) if, in addition to the agent(s) indicated in Box No. IV, there are further agents: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;
 - (v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "patent of addition," or "certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "continuation" or "continuation-in-part": in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;
 - (vi) if, in Box No. VI, there are more than five earlier applications whose priority is claimed: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.
2. If, with regard to the precautionary designation statement contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of this statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.

CONTINUATION OF BOX VI:

This application claims priority of US patent application no. 60/253,785, filed on 29 November 2000 (29.11.00) and priority of US patent application no. 60/253, 787, filed on 29 November 2000 (29.11.00).

See Notes to the request form

Box No. VIII (iv) **DECLARATION: INVENTORSHIP** (only for the purposes of the designation of the United States of America)
The declaration must conform to the following standardized wording provided for in Section 11.4: see Notes to Boxes Nos. VII, VIII (i) to (iv)
in general and the specific Notes to Box No. VIII (iv). If this Box is not used, this sheet should not be included in the request.

**Declaration of Inventorship (Rules 4.17(iv) and 51bis.1(a)(iv))
for the purposes of the designation of the United States of America:**

I hereby declare that I believe I am the original, first and sole (if only one inventor is listed below) or joint (if more than one inventor is listed below) inventor of the subject matter which is claimed and for which a patent is sought.

This declaration is directed to the international application of which is Form a part (if filing declaration with application).

This declaration is directed to international application No. PCT/US01/45023 (if furnishing declaration pursuant to Rule 26ter).

I hereby declare that my residence, mailing address, and citizenship are as stated next to my name.

I hereby state that I have reviewed and understood the contents of the above-identified international application, including the claims of said application. I have identified in the request of said application, in compliance with PCT Rule 4.10, any claim to foreign priority, and I have identified below, under the heading "Prior Applications," by application number, country or Member of the World Trade Organization, day, month and year of filing, any application for a patent or inventor's certificate filed in a country other than the United States of America, including any PCT international application designating at least one country other than the United States of America, having a filing date before that of the application on which foreign priority is claimed.

Prior Applications: US 60/253,785, US 60/253,787, both Filed 29 November 2000

I hereby acknowledge the duty to disclose information that is known by me to be material to patentability as defined by 37 C.F.R. § 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the PCT international filing date of the continuation-in-part application.

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.

Name: George E. Seidel

Residence: 3101 Arrowhead Road, Colorado 80535 USA
(city and either US state, if applicable, or country)

Mailing Address: 3101 Arrowhead Road
Colorado 80535 USA

Citizenship: United States of America

Inventor's Signature: George E. Seidel
(if not contained in the request, or if declaration is corrected or added under Rule 26ter after the filing of the international application. The signature must be that of the inventor, not that of the agent)

Date: December 5, 2001
(of signature which is not contained in the request, or of the declaration that is corrected or added under Rule 26ter after the filing of the international application)

Name: Tae Kwang Suh

Residence: 2044 Glenmoor Drive, Fort Collins, Colorado 80521 USA
(city and either US state, if applicable, or country)

Mailing Address: 2044 Glenmoor Drive
Fort Collins, Colorado 80521 USA

Citizenship: South Korea

Inventor's Signature: Tae Kwang Suh
(if not contained in the request, or if declaration is corrected or added under Rule 26ter after the filing of the international application. The signature must be that of the inventor, not that of the agent)

Date: December 5, 2001
(of signature which is not contained in the request, or of the declaration that is corrected or added under Rule 26ter after the filing of the international application)

☒ This declaration is continued on the following sheet, "Continuation of Box No. VIII (iv)".

Continuation of Form No. VIII (i) to (v) **DECLARATION**

(If the space is insufficient to carry out Form No. VIII (i) to (v) as prescribed, including in the case where more than one inventor are to be named in Form No. VIII (i), in such case, write "Continuation of Form No. VIII..." (indicate the form number of the Form) and furnish the information on the same manner as required for the purpose of the form in which the space was insufficient. If additional space is needed in respect of two or more declarations, a separate continuation sheet must be used for each such declaration. If this form is not used, this sheet should not be included in the request.)

Name: Kehuan Lu

Residence: 14 Burrough Field

Torrington, Cambridge CB4 9NW

United Kingdom

Mailing Address: 14 Burrough Field

Torrington, Cambridge CB4 9NW

United Kingdom

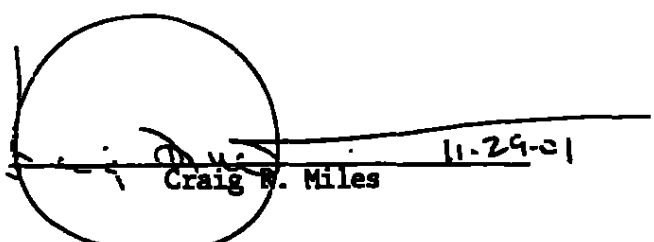
Citizenship: United Kingdom

Inventor's Signature: 

Date: November 11, 2001

Feb 11, 2002

Box No. IX CHECK LIST: LANGUAGE OF FILING		
This international application contains: (a) the following number of sheets in paper form: request (including declaration sheets) : 3 description (excluding sequence listing part) : 31 claims : 15 abstract : 1 drawings : 3 Sub-total number of sheets : 58 sequence listing part of description (actual number of sheets if filed in paper form, whether or not also filed in computer readable form: see (b) below) : --- Total number of sheets : 58 (b) sequence listing part of description filed in computer readable form (i) ☐ only (under Section 801(a)(i)) (ii) ☐ in addition to being filed in paper form (under Section 801(a)(ii)) Type and number of carriers (diskette, CD-ROM, CD-R or other) on which the sequence listing part is contained (additional copies to be indicated under item 9(II), in right column):	This international application is accompanied by the following items (mark the applicable check-boxes below and indicate in right column the number of each item): 1. ☒ fee calculation sheet : 1 2. ☒ original separate power of attorney (2) and Power of Agent (2) : 4 3. ☐ original general power of attorney : 1 4. ☐ copy of general power of attorney; reference number, if any: : 1 5. ☐ statement explaining lack of signature : 1 6. ☐ priority document(s) identified in Box No. VI as item(s): : 1 7. ☐ translation of international application into (language): : 1 8. ☐ separate indications concerning deposited microorganism or other biological material : 1 9. ☐ sequence listing in computer readable form (indicate also type and number of carriers (diskette, CD-ROM, CD-R or other)) (i) ☐ copy submitted for the purposes of international search under Rule 13ter only (and not as part of the international application) : 1 (ii) ☐ (only where check-box (b)(i) or (b)(ii) is marked in left column) additional copies including, where applicable, the copy for the purposes of international search under Rule 13ter : 1 (iii) ☐ together with relevant statement as to the identity of the copy or copies with the sequence listing part mentioned in left column : 1 10. ☒ other (specify): USPO Letter of Transmittal; Mailing Certificates; Letter of Transmittal : 1	Number of items
Figure of the drawings which should accompany the abstract: Fig. 2	Language of filing of the international application: English	

Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE	
Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).	
 Craig R. Miles 11-29-01	

For receiving Office use only	
1. Date of actual receipt of the purported international application:	2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 1(2):	
5. International Searching Authority (if two or more are competent): ISA /	6. ☐ Transmittal of search copy delayed until search fee is paid

For International Bureau use only
Date of receipt of the record copy by the International Bureau:

This sheet is not part of and does not count as a sheet of the international application.

PCT

FEE CALCULATION SHEET

Annex to the Request

For receiving Office use only

International Application No.

Date stamp of the receiving Office

Applicant's or agent's
file reference

XYFrozSorPCT

Applicant

Colorado State University through its agent
XY, Inc. and Colorado State University Research Foundation

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE 240.00 T

2. SEARCH FEE 700.00 S

International search to be carried out by _____
(If two or more International Searching Authorities are competent to carry out the international
search, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

Where item (b) of Box No. IX applies, enter Sub-total number of sheets } _____
Where item (b) of Box No. IX does not apply, enter Total number of sheets }

b1 first 30 sheets 382.00 b1

b2 28 x 9.00 = 252.00 b2
number of sheets in excess of 30 fee per sheet

b3 additional component (only if sequence listing part of description
is filed in computer readable form under Section 801(a)(i), or
both in that form and on paper, under Section 801(a)(ii):

400 x _____ = _____ b3
fee per sheet

Add amounts entered at b1, b2 and b3 and enter total at B 634.00 B

Designation Fees

The international application contains _____ designations.

6 x 82.00 = 492.00 D
number of designation fees payable (maximum 6) amount of designation fee

Add amounts entered at B and D and enter total at I 1126.00 I

(Applicants from certain States are entitled to a reduction of 75% of the
international fee. Where the applicant is (or all applicants are) so entitled, the total
to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT (if applicable) 30.00 P

5. TOTAL FEES PAYABLE 2096.00

Add amounts entered at T, S, I and P, and enter total in the TOTAL box

TOTAL

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

☐ authorization to charge deposit account (see below) ☐ postal money order ☐ cash ☐ coupons
☒ cheque ☐ bank draft ☐ revenue stamps ☐ other (specify): _____

AUTHORIZATION TO CHARGE (OR CREDIT) DEPOSIT ACCOUNT

(This mode of payment may not be available at all receiving Offices)

☐ Authorization to charge the total fees indicated above.
☐ (This check-box may be marked only if the conditions for deposit accounts
of the receiving Office so permit.) Authorization to charge any deficiency
or credit any overpayment in the total fees indicated above.
☐ Authorization to charge the fee for priority document.

Receiving Office: RO/ _____

Deposit Account No.: _____

Date: _____

Name: _____

Signature: _____

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY -

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT
OR THE DECLARATION

(PCT Rule 44.1)

To: CRAIG R. MILES
SANTANGELO LAW OFFICES, P.C.
125 SOUTH HOWES, THIRD FLOOR
FORT COLLINS, COLORADO 80521

Date of Mailing
(day/month/year) 03 JUL 2002

Applicant's or agent's file reference
XYFROZSORPCT

FOR FURTHER ACTION See paragraphs 1 and 4 below

International application No.
PCT/US01/45023

International filing date
(day/month/year) 29 NOVEMBER 2001

Applicant
XY, INC.

1. ☒ The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (+1-22) 740.14.85

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(s)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. Further action(s): The applicant is reminded of the following:

Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 bis 1 and 90 bis 3, respectively, before the completion of the technical preparations for international publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20531

Facsimile No. (703) 306-9930

Authorized officer

PETER PARAS JR

Telephone No. (703) 308-0188

Form PCT/ISA/680 (July 1988)*

(See notes on accompanying sheet)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference XYFROZSORPCT	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/US01/48088	International filing date (day/month/year) 20 NOVEMBER 2001	(Earliest) Priority Date (day/month/year) 20 NOVEMBER 2000
Applicant XY, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 2 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 26.1(b)).

b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2. ☐ Certain claims were found unsearchable (See Box I).

3. ☐ Unity of invention is lacking (See Box II).

4. With regard to the title,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.3(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No. _____

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/45093

28

A. CLASSIFICATION OF SUBJECT MATTER IPC(7) : C12N 5/00, 5/08, 15/00; C12M 1/00; A01K 67/00, 67/033 US CL : 435/925, 925.1, 288.5; 800/8, 21 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/925, 925.1, 288.5; 800/8, 21 Documentation searched other than minimum documentation to the extent that such documents are included in the field searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WEST, MEDLINE, BIOSIS, EMBASE, SCISEARCH, CAPLUS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim
Y	US 5,439,362 A (SPAULDING) 08 August 1995, see entire document.	1-75
Y	US 5,780,230 A (LI et al) 14 July 1998, see entire document.	1-75
Y	US 5,919,621 A (BROWN) 06 July 1999, see entire document.	1-75
Y	US 6,071,689 A (SEIDEL et al) 06 June 2000, see entire document.	1-75
Y	US 6,149,867 A (SEIDEL et al) 21 November 2000, see entire document.	1-75
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
*	Special categories of cited documents	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to amend the principle or theory underlying the invention "X" document of particular relevance; the claimed invention appears considered novel or cannot be considered to involve an invention when the document is taken alone "Y" document of particular relevance; the claimed invention appears considered to involve an inventive step when the document is viewed with one or more other such documents, such combination is obvious to a person skilled in the art "A" document published prior to the international filing date but later than the priority date claimed
"A"	document defining the general state of the art which is not considered to be of particular relevance	
"E"	earlier document published on or after the international filing date	
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O"	document referring to an oral disclosure, use, exhibition or other means	
"P"	document published prior to the international filing date but later than the priority date claimed	
Date of the actual completion of the international search 01 JUNE 2002		Date of mailing of the international search report 03 JUL 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-9850		Authorized officer PETER PARAS JR Telephone No. (703) 308-6340 <i>Janice Fort</i>

The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below:

IPEA/ US

PCT**CHAPTER II****DEMAND**

under Article 31 of the Patent Cooperation Treaty.
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only	
Identification of IPEA	Date of receipt of DEMAND
Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION	
Applicant's or agent's file reference XYFrozSorPCT	
International application No. PCT/US01/45023	International filing date (day/month/year) 29 November 2001
(Earliest) Priority date (day/month/year) 29 November 2000	
Title of invention System To Separate Frozen-Thawed Spermatozoa Into X-Chromosome Bearing And Y-Chromosome Bearing Populations	
Box No. II APPLICANT(S)	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)	
XY, Inc. 1108 North Lemay Avenue Fort Collins, Colorado 80524 United States of America	
Telephone No. (970) 493-3113	
Facsimile No. (970) 493-3114	
Teleprinter No.	
Applicant's registration No. with the Office	
State (that is, country) of nationality: US	State (that is, country) of residence: US
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)	
COLORADO STATE UNIVERSITY through its agent Colorado State University Research Foundation 601 South Howes, Room 410 Fort Collins, Colorado 80525 United States of America	
State (that is, country) of nationality: US	State (that is, country) of residence: US
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)	
SEIDEL, George E. 3101 Arrowhead Road Fort Collins, Colorado 80535 United States of America	
State (that is, country) of nationality: US	State (that is, country) of residence: US
☒ Further applicants are indicated on a continuation sheet.	

Sheet No. 2.

International application No.
PCT/US01/45023

Continuation of Box No. II APPLICANT(S)

If none of the following sub-headers is used, this sheet should not be included in the demand.

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

SUH, Tae Kwang
2044 Glenmoor Drive
Fort Collins, Colorado 80521
United States of America

State (that is, country) of nationality:
KR

State (that is, country) of residence:
US

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

LU, Kehaun
14 Burrough Field
Impington, Cambridge CB4 9NW
United Kingdom

State (that is, country) of nationality:
UK

State (that is, country) of residence:
UK

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

State (that is, country) of nationality:

State (that is, country) of residence:

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

State (that is, country) of nationality:

State (that is, country) of residence:

☐ Further applicants are indicated on another continuation sheet.

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE	
The following person is ☒ agent ☐ common representative and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination. ☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked. ☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.	
Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)</i> MILES, Craig R. Santangelo Law Offices, P.C. 125 South Howes, Third Floor Fort Collins, CO 80521 United States of America	Telephone No. 970-224-3100
	Facsimile No. 970-224-3175
	Teleprinter No.
	Agent's registration No. with the Office 45,954
☐ Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.	
Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION	
Statement concerning amendments:*	
1. The applicant wishes the international preliminary examination to start on the basis of: ☒ the international application as originally filed the description ☒ as originally filed ☐ as amended under Article 34 the claims ☒ as originally filed ☐ as amended under Article 19 (together with any accompanying statement) ☐ as amended under Article 34 the drawings ☒ as originally filed ☐ as amended under Article 34	
2. ☐ The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.	
3. ☐ The applicant wishes the start of the international preliminary examination to be postponed until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). <i>(This check-box may be marked only where the time limit under Article 19 has not yet expired.)</i>	
* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.	
Language for the purposes of international preliminary examination: <u>English</u> ☒ which is the language in which the international application was filed. ☐ which is the language of a translation furnished for the purposes of international search. ☐ which is the language of publication of the international application. ☐ which is the language of the translation (to be) furnished for the purposes of international preliminary examination.	
Box No. V ELECTION OF STATES	
The applicant hereby elects all eligible States <i>(that is, all States which have been designated and which are bound by Chapter II of the PCT)</i> excluding the following States which the applicant wishes not to elect: None	

Sheet No. 4-

International application No.
PCT/US01/45237

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:

- | | | |
|---|---|--------|
| 1. translation of international application | : | sheets |
| 2. amendments under Article 34 | : | sheets |
| 3. copy (or, where required, translation) of amendments under Article 19 | : | sheets |
| 4. copy (or, where required, translation) of statement under Article 19 | : | sheets |
| 5. letter | : | sheets |
| 6. other (specify) Firm Letter of Transmittal and Express Mail Certificates for each document | : | sheets |

For International Preliminary
Examining Authority use only
received not received

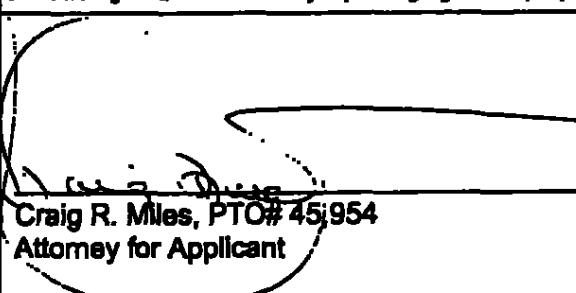
☐	☐
☐	☐
☐	☐
☐	☐
☐	☐
☐	☐

The demand is also accompanied by the item(s) marked below:

- | | |
|--|--|
| 1. ☒ fee calculation sheet | 5. ☐ statement explaining lack of signature |
| 2. ☐ original separate power of attorney | 6. ☐ sequence listing in computer readable form |
| 3. ☐ original general power of attorney | 7. ☐ other (specify): |
| 4. ☐ copy of general power of attorney; reference number, if any: | |

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

 Craig R. Miles, PTO# 45,954 Attorney for Applicant	<u>06-27-02</u> Date
--	-------------------------

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:	
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):	
3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply.	☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.	
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.	

For International Bureau use only

Demand received from IPEA on:

PATENT COOPERATION TREATY

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From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
CRAIG R. MILES
SANTANGELO LAW OFFICES, P.C.
125 SOUTH HOWES
THIRD FLOOR
FORT COLLINS, CO 30521

PCT

WRITTEN OPINION

(PCT Rule 66)

Applicant's or agent's file reference XYFROZSORPCT		Date of Mailing (day/month/year) 16 APR 2003
International application No. PCT/US01/45023		REPLY DUE within 1 month/days from the above date of mailing
International filing date (day/month/year) 29 November 2001 (29.11.2001)	Priority date (day/month/year) 29 November 2000 (29.11.2000)	
International Patent Classification (IPC) or both national classification and IPC IPC(7): C12N 5/00, 5/02, 15/00; C12M 1/00; A01K 67/00, 67/033 and US Cl.: 435/325, 283.1, 288.5; 800/8, 21		
Applicant XY, INC.		

1. This written opinion is the first (first, etc.) drawn by this International Preliminary Examining Authority.
2. This opinion contains indications relating to the following items:
 - I ☒ Basis of the opinion
 - II ☐ Priority
 - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
 - IV ☐ Lack of unity of invention
 - V ☒ Reasoned statement under Rule 66.2 (a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
 - VI ☐ Certain documents cited
 - VII ☐ Certain defects in the international application
 - VIII ☐ Certain observations on the international application
3. The applicant is hereby invited to reply to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension. See rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4.
 For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
 For an informal communication with the examiner, see Rule 66.6

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.
4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 29 March 2003 (29.03.2003).

Name and mailing address of the IPEA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20531
 Facsimile No. (703)303-3230

Authorized officer
 Peter Paras, Jr. *Janice Foy*
 Telephone No. 703-308-1123

WRITTEN OPINION

International application No.

PCT/US01/49023

I. Basis of the opinion

1. With regard to the elements of the international application:*

- ☒ the international application as originally filed
- ☒ the description:
 pages 1-31 _____, as originally filed
 pages NONE _____, filed with the demand
 pages NONE _____, filed with the letter of _____
- ☒ the claims:
 pages 32-46 _____, as originally filed
 pages NONE _____, as amended (together with any statement) under Article 19
 pages NONE _____, filed with the demand
 pages NONE _____, filed with the letter of _____
- ☒ the drawings:
 pages 1-3 _____, as originally filed
 pages NONE _____, filed with the demand
 pages NONE _____, filed with the letter of _____
- ☐ the sequence listing part of the description:
 pages NONE _____, as originally filed
 pages NONE _____, filed with the demand
 pages NONE _____, filed with the letter of _____

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
 These elements were available or furnished to this Authority in the following language _____ which is:

- ☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the written opinion was drawn on the basis of the sequence listing:

- ☐ contained in the international application in printed form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

- ☐ the description, pages NONE _____
- ☐ the claims, Nos. NONE _____
- ☐ the drawings, sheets/fig NONE _____

5. ☐ This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed."

WRITTEN OPINION

International application No.

PCT/US01/45023

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The question whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application,
- ☒ claims Nos. 10-12, 20-22, 24-39, 48-56, 67-70 and 75

because:

- ☐ the said international application, or the said claim Nos. _____ relate to the following subject matter which does not require international preliminary examination (specify):

- ☒ the description, claims or drawings (indicate particular elements below) or said claims Nos. 10-12, 20-22, 24-39, 48-56, 67-70 and 75 are so unclear that no meaningful opinion could be formed (specify):

Claims 10-12, 20-22, 30-39, 48-52, 54-56, 67-70 and 75 are improper multiple dependent claims as per PCT Rule 6.4(a). Claims 24-29 and 53-56 are unclear because they are directed to different methods than the methods from which they depend.

- ☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed.
- ☐ no international search report has been established for said claims Nos. _____.

2. A written opinion cannot be drawn due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

- ☐ the written form has not been furnished or does not comply with the standard.
- ☐ the computer readable form has not been furnished or does not comply with the standard.

WRITTEN OPINION

International application No.
PCT/US01/45023

V. Reasoned statement under Rule 66.2(a)(II) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims <u>NONE</u>	YES
	Claims <u>1-9, 13-19, 23, 40-47, 57-66, and 71-74</u>	NO
Inventive Step (IS)	Claims <u>NONE</u>	YES
	Claims <u>1-9, 13-19, 23, 40-47, 57-66, and 71-74</u>	NO
Industrial Applicability (IA)	Claims <u>1-9, 13-19, 23, 40-47, 57-66, and 71-74</u>	YES
	Claims <u>NONE</u>	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-9, 13-19, and 40-47 lack novelty under PCT Article 33(2) as being anticipated by Schenk et al. Schenk et al teach staining of bovine spermatozoa with Hoechst 33342 dye. See page 376. Schenk et al further teach sorting of the stained spermatozoa with a flow cytometer to separate sperm having X and Y chromosomes. See throughout the entire document. Schenk et al also teach staining of previously frozen spermatozoa with Hoechst dye. See pages 1380-1388. The flow cytometer used by Schenk anticipates the claimed cytometer as both cytometers appear to be capable of sorting spermatozoa stained by Hoechst dye under the same conditions. Thus, the teachings of Schenk et al anticipate all of the instant claim limitations.

Claims 1-9, 13-19, 23, 40-47, 57-66 and 71-74 lack novelty under PCT Article 33(2) as being anticipated by Seidel et al. Seidel et al teach methods of producing bovine embryos having a predetermined sex comprising using sperm that had been previously stained with Hoechst dye and sorted by flow cytometry as per the methodology of Schenk as discussed above. See page 1408, where the method of Schenk et al is referenced. Thus, the teachings of Seidel et al anticipate all of the instant claim limitations.

NEW CITATIONS

SEIDEL G.E. et al. Insemination of heifers with sexed sperm. Theriogenology. 1999, Vol. 52, pages 1407-1420, see entire document.

SCHENK J.L. et al. Cryopreservation of flow-sorted bovine spermatozoa. Theriogenology. 1999, Vol. 52, pages 1375-1391, see entire document.

WRITTEN OPINION

International application No.
PCT/US01/43023

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

TIME LIMIT:

The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.434(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.

DISPOSICIÓN PARA SEPARAR ESPERMATOZOIDES CONGELADOS EN POBLACIONES QUE CONTIENEN CROMOSOMAS X Y CROMOSOMAS Y

I. CAMPO TÉCNICO

La invención se refiere a la unión substancialmente uniforme de fluorocromo (s) al ADN dentro de espermatozoides de mamíferos (o células de esperma) que permiten que tales espermatozoides etiquetados sean separados en poblaciones de alta pureza que contienen cromosomas X y que contienen cromosomas Y. Específicamente, se refiere a métodos para unir en forma substancialmente uniforme fluorocromo (s) al ADN de espermatozoides de mamíferos contenidos dentro del semen previamente congelados y luego descongelados. Además, la invención se refiere adicionalmente a dispositivos métodos y composiciones para el uso de poblaciones de espermatozoides separados en grupos que contienen cromosomas X y grupos que contienen cromosomas Y, de alta pureza, y que han sido separados de semen previamente congelado y descongelado en procedimientos que comprenden, aunque no están limitados a esto, inseminación artificial, inseminación quirúrgica, y fertilización invitro y técnicas de cultivo embrionario.

ESTADO DEL ARTE DE LA INVENCION

El esperma puede ser recolectado a partir de una gran variedad de mamíferos y luego puede ser separado en poblaciones que contienen cromosomas x y poblaciones que contienen cromosomas Y, basadas en la diferencia en el contenido de ADN. En algunos métodos convencionales de separación de espermatozoides, el contenido de ADN de los espermatozoides a ser separados puede ser teñido o coloreado con fluorocromo (s) que frente a una excitación emite (s) una cantidad medible de fluorescencia. Debido a que los espermatozoides que contienen cromosomas X tienen una mayor

cantidad de ADN que los espermatozoides que contienen cromosomas Y, cada espermatozoide que contiene cromosomas X tiene la capacidad de unirse a una cantidad relativamente mayor de fluorocromo que el espermatozoide correspondiente que contiene cromosomas Y. La comparación de la magnitud relativa de la fluorescencia emitida ante la excitación del fluorocromo (s) permita la aislamiento de los espermatozoides portadores de cromosomas X de aquellos espermatozoides que son portadores de cromosomas Y, tal como se describe en la patente de los Estados Unidos de Norteamérica N° 5,135,759 que se incorpora a la presente a modo de referencia.

Aún cuando los espermatozoides que contienen cromosomas X y los espermatozoides que contienen cromosomas Y han sido diferenciados por, y separados en base a, la diferencia en la fluorescencia emitida, durante muchos años, y aún cuando hay un gran mercado comercial para las poblaciones aisladas de espermatozoides que contienen cromosomas X y de espermatozoides que contienen cromosomas Y, todavía persisten problemas significativos que deben ser resueltos.

Un problema significativo con los métodos convencionales de separación de espermatozoides que contienen cromosomas X de espermatozoides que contienen cromosomas Y puede ser que cada población resultante contenga un número significativo de espermatozoides separados incorrectamente que pertenezcan a la otra población. Este problema en la diferenciación entre los espermatozoides puede, en parte, ser atribuido a la falta de uniformidad en la cantidad de fluorocromo que se adhiere al ADN de los espermatozoides. Como tal, se genera un rango en la cantidad de fluorocromo que se adhiere a los espermatozoides que contienen cromosomas X y se genera un rango en la cantidad de fluorocromo que se

adhiere a los espermatozoides que contienen cromosomas Y. Cuando éstos rangos en la cantidad de fluorocromo superan o dan como resultados algunos valores que son muy similares, puede ser difícil o imposible clasificar aquellos espermatozoides individuales como correspondientes a una población o la otra, con cualquier grado de certeza y puede suceder una contaminación cruzada de las poblaciones.

Este particular problema puede verse exacerbado con respecto a los espermatozoides obtenidos a partir de semen de mamíferos congelado y subsecuentemente descongelado o derretido. La pureza media para la población de espermatozoides separada que contiene cromosomas Y, derivada de semen previamente congelado y descongelado puede ser del 85% o menos, y la pureza media para una población separada de espermatozoides que contienen cromosomas X, derivada de semen previamente congelado y descongelado, puede ser del 82% o menos.

Otro problema significativo que esta asociado con la coloración del ADN de los espermatozoides puede ser los problemas perjudiciales en los rangos de fertilización y el subsecuente desarrollo embrionario de oocito (s) (oocito, oótide, óvulo o una pluralidad de los mismos, como pueda resultar apropiado dentro de una aplicación específica). Un aspecto de éste problema puede ser que la cantidad de tintura o colorante adherido al ADN puede afectar la viabilidad de los espermatozoides dando como resultado rangos de fertilización inferiores. Otro aspecto de éste problema puede ser la cantidad de tiempo que pasa durante el teñido o coloración del ADN puede afectar la viabilidad del esperma que da como resultado rangos inferiores de fertilización. Otro aspecto de éste problema puede ser que la cantidad de tiempo que pasa durante la coloración o teñido del ADN puede

disminuir los rangos de la subsecuente segmentación de los oocitos fertilizados con dichos espermatozoides teñidos o coloreados. Se ha observado un 20% de declinación en los rangos de segmentación para los oocitos cuando los tiempos de tinción o coloración requieren 190 minutos, en comparación con lo que sucede cuando los tiempos de coloración requieren de 60 minutos. Otro aspecto de éste problema puede ser que el porcentaje de oocitos fertilizados con espermatozoides teñidos pueda resultar en una blastulación inferior, tal como se describe en el artículo de la publicación titulado "fertilización in-vitro con espermatozoides seleccionados con citómetro de flujo", Theriogenology 52: 1393-1405 (1999), que se incorpora a la presente a modo de referencia.

Otro problema significativo puede ser que el espermatozoide criopreservado puede demostrar una capacitación, mayor, y el lapso de tiempo en que tal espermatozoide es viable se puede ver acortado. Por esto, si los espermatozoides previamente congelados deben ser separados en poblaciones que contienen cromosomas X y poblaciones que contienen cromosomas Y que luego han de ser utilizadas en aplicaciones tales como fertilización in-vitro, inseminación artificial in-vivo, o lo similar, entonces los procedimientos rutinarios de coloración pueden tener que ser abreviados para mantener un número adecuado de células de espermatozoides viables.

En relación con los problemas de la coloración uniforme de los espermatozoides, aun cuando los espermatozoides son obtenidos a partir de semen congelado-descongelado; con el mantenimiento de la viabilidad del espermatozoide; la separación de los espermatozoides coloreados en poblaciones que contienen cromosomas X y poblaciones que contienen cromosomas Y, aun cuando los espermatozoides que son separados son obtenidos a partir de semen previamente

congelado; la generación de poblaciones de espermatozoides que contienen cromosomas X y espermatozoides que contienen cromosomas Y, con alta pureza; y la utilización exitosa de espermatozoides separados para inseminación artificial, inseminación quirúrgica, y técnicas de fertilización in-vitro, puede entenderse que existen problemas significativos con la tecnología convencional, los cuales son tratados por la presente invención para su resolución.

DESCRIPCIÓN DE LA INVENCION

Un amplio objeto de las realizaciones de la invención puede ser proveer una tecnología de coloración de ADN que permita que cantidades sustancialmente uniformes de fluorocromo se adhieran o se unan al ADN de espermatozoides individuales que contienen cromosomas X y cantidades sustancialmente uniformes de fluorocromo se adhieran a espermatozoides individuales que contienen cromosomas Y, dentro de una cantidad de semen.

Un aspecto de este amplio objeto de la invención puede ser el de estrechar el rango de magnitud de fluorescencia emitida para cada una de las poblaciones que contienen cromosomas X y cromosomas Y, de espermatozoides, al pasar a través de una fuente de excitación de fluorocromo.

Otro aspecto de este amplio objeto de la invención puede ser aumentar la diferencia entre los valores medios de magnitud de la fluorescencia emitida para cada una de las poblaciones, es decir la población que contiene cromosomas X y la población que contiene cromosomas Y, de espermatozoides, al pasar a través de una fuente de excitación de fluorocromo.

Otro aspecto de este amplio objeto de la invención puede ser disminuir el numero de espermatozoides incorrectamente asignados a cada una de las poblaciones de

espermatozoides, es decir la población que contiene cromosomas X y la población que contiene cromosomas Y.

Otro aspecto de este amplio objeto de la invención puede ser generar poblaciones separadas que contienen cromosomas X y cromosomas Y, que tengan una pureza mayor del 85%, o una pureza mayor del 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, o aun 99%.

Otro amplio objeto de la realización de la invención puede ser permitir la determinación de una genética de alto rango. En lugar de limitarse a la genética de individuos provenientes de especies de mamífero que tengan proximidad a una instalación de selección o separación de espermatozoides, la genética que represente una amplia variedad de individuos de numerosas especies puede ser transportada como semen congelado a instalaciones distantes de separación de espermatozoides para la subsiguiente separación en poblaciones que contienen cromosomas X y poblaciones que contienen cromosomas Y. Estas especies de mamíferos pueden incluir, aunque no están limitadas a los mismos, primates, tales como chimpancés, gorilas, humanos, o lo similar; mamíferos marinos, tales como ballenas, marsopas y delfines, o los similares; bóvidos, ovinos, cerdos, felinos; o equinos, sólo a modo de pocos ejemplos. También se puede incluir los materiales genéticos que son considerados raros porque las especies de mamíferos pueden estar en peligro o pueden comprender un pequeño numero de animales; o considerados raros porque los individuos tienen deseables atributos morfológicos, fisiológicos o intelectuales.

Otro amplio objeto de las realizaciones de la invención puede proveer la tecnología de separación para la diferenciación entre espermatozoides que contienen cromosomas

X y espermatozoides que contienen cromosomas Y, obtenidos a partir de semen congelado y luego descongelado.

Otro objeto de las realizaciones de la invención puede ser proveer una tecnología de coloración o tinción de ADN para colorear mas uniformemente el ADN de espermatozoides contenidos en el semen congelado-descongelado para mejorar la resolución aparente ente los espermatozoides que contienen cromosomas X y espermatozoides que contienen cromosomas Y.

Otro objeto de las realizaciones de la invención puede ser proveer muestras de inseminación artificial de alta pureza preparadas a partir de espermatozoides separados de semen congelado-descongelado.

Otro objeto de las realizaciones de la invención puede ser proveer muestras de inseminación artificial de alta pureza y de bajas dosis, preparadas a partir de espermatozoides separados obtenidos de semen congelado-descongelado.

Otro objeto de las realizaciones de la invención puede ser proveer muestras de inseminación de alta pureza para procedimientos de inseminación quirúrgica, preparados a partir de espermatozoides separados que se obtienen de semen congelado-descongelado.

Otro objeto de una realización de la invención puede ser proveer muestras de inseminación de alta pureza para procedimientos de fertilización in-vitro, preparados a partir de espermatozoides separados de semen congelado-descongelado.

Otro objeto de una realización de la invención puede ser proveer muestras de inseminación de alta pureza, para procedimientos de fertilización in-vitro, preparados a partir de espermatozoides separados y provenientes humano congelado-descongelado.

Otro objeto de una realización de la invención puede ser proveer tecnología para la coloración y separación de espermatozoides a partir de esperma congelado-descongelado, en poblaciones que contienen cromosomas X y poblaciones que contienen cromosomas Y, para fertilización in-vitro de oocitos que no es perjudicial para los rangos de segmentación o desarrollo embrionario.

Naturalmente, objetos adicionales de la invención se describen a lo largo de otras áreas de la memoria descriptiva.

BREVE DESCRIPCIÓN DE LOS DIBUJOS

La Figura 1 muestra una realización particular de la invención para la coloración de ADN de espermatozoides contenidos en semen congelado-descongelado (es decir, congelado y luego descongelado).

La Figura 2 muestra una realización particular de la invención para la separación de espermatozoides a partir de semen congelado-descongelado en espermatozoides que contienen cromosomas X y espermatozoides que contienen cromosomas Y.

La Figura 3 muestra una vista adicional de una realización particular de la invención para la separación de espermatozoides a partir de semen congelado-descongelado en espermatozoides que contienen cromosomas X y espermatozoides que contienen cromosomas Y.

MODO(S) PARA LLEVAR A CABO LA INVENCION

Para separa rutinariamente espermatozoides (vivos, fijos, viables, no viables, o núcleos) en muestras de alta pureza que contienen cromosomas X y muestras de alta pureza

que contienen cromosomas Y, el método utilizado para separar los espermatozoides que contienen cromosomas X de los espermatozoides que contienen cromosomas Y debe proveer una suficiente resolución de los espermatozoides que contienen cromosomas X con respecto a los espermatozoides que contienen cromosomas Y de manera que la etapa o etapas de separación o clasificación se consigan sin una contaminación cruzada substancial.

La resolución o diferenciación de los espermatozoides se puede basar en la determinación de la diferencia en la emisión fluorescente de la cantidad de fluorocromo adherido al ADN dentro de los espermatozoides que contienen cromosomas X, frente a la excitación, y la emisión de fluorescencia proveniente de la cantidad de fluorocromo adherido al ADN dentro de los espermatozoides que contienen cromosomas Y, también frente a la excitación. La separación de los espermatozoides que contienen cromosomas X y los espermatozoides que contienen cromosomas Y en base a esta diferencia mensurable puede luego ser lograda por medio de una variedad de métodos tales como citometría de flujo, cromatografía líquida, electroforesis de gel, y otras tecnologías que comparan de manera similar la magnitud relativa de fluorescencia para diferenciar entre los espermatozoides que contienen cromosomas X y los espermatozoides que contienen cromosomas Y.

Las disposiciones de separación de espermatozoides pueden tener problemas en diferenciar entre la emisión fluorescente generada por el fluorocromo adherido al ADN de los espermatozoides X y la emisión fluorescente generada por el fluorocromo adherido al ADN de los espermatozoides Y frente a la excitación cuando la cantidad de fluorocromo adherido al ADN de los espermatozoides individuales no es

consistente dentro de las poblaciones que contienen cromosomas Y y las poblaciones que contienen cromosomas X. Estas dificultades de diferenciación entre la cantidad de emisiones fluorescentes generadas por el fluorocromo o los fluorocromos adheridos resulta exacerbada los espermatozoides son obtenidos a partir de esperma congelado-descongelado que está coloreado por medio de técnicas convencionales.

La falla en la coloración del ADN de los espermatozoides puede consistentemente generar un rango más amplio de especies fluorescentes para las poblaciones de espermatozoides que contienen cromosomas X y las que contienen cromosomas Y. Este rango más amplio de especies fluorescentes para las dos poblaciones da como resultado un rango aumentado de pesos moleculares aparente de ADN y una capacidad disminuida de resolver los espermatozoides que contienen cromosomas X y los espermatozoides que contienen cromosomas Y. La disminución en la resolución hace que la separación entre los espermatozoides que contienen cromosomas X y los espermatozoides que contienen cromosomas Y sea más difícil y resulte en una contaminación cruzada entre las poblaciones y se obtengan muestras separadas de espermatozoides con pureza más baja.

Las realizaciones particulares de la invención proveen una tecnología para teñir o colorear el ADN de espermatozoides vivos viables o el ADN de los espermatozoides provenientes de especímenes de semen congelado-descongelado para permitir una mayor resolución de los espermatozoides que contienen cromosomas X y de los espermatozoides que contienen cromosomas Y, dando como resultado poblaciones de células de esperma de alta pureza que contienen cromosomas X y que contienen cromosomas Y. Como tal, se entiende que el término alta pureza puede significar una mayor resolución de los

espermatozoides que contienen cromosomas X con respecto a los espermatozoides que contienen cromosomas y, en comparación con las tecnologías de coloración convencionales para una aplicación dada. La alta pureza también puede significar una contaminación cruzada menor entre las poblaciones separadas de espermatozoides comparadas con las tecnologías convencionales de separación.

Por ejemplo, en particular las realizaciones de citometría de flujo de acuerdo con la invención, la alta pureza para espermatozoides vivos teñidos congelados-descongelados puede significar poblaciones seleccionadas de espermatozoides que contienen cromosomas X y espermatozoides que contienen cromosomas Y que tienen una pureza mayor de alrededor del 85%. Sin embargo, si se están seleccionando núcleos de esperma o esperma viable vivo, la alta pureza puede significar ovulaciones que contienen cromosomas X y poblaciones que contienen cromosomas y, que contienen una pureza mayor de alrededor del 90%. Tal como se puede entender, la definición de alta pureza involucra en contexto, una comparación de los resultado obtenidos de cada realización de la invención comparados con los resultados obtenidos cuando se utilizan tecnologías convencionales para una aplicación particular. En el contexto de los espermatozoides que tienen ADN que se tiñen en forma pobre, tal como el ADN de los espermatozoides congelados-descongelados anteriormente citados, la alta pureza puede significar poblaciones de espermatozoides aislados que tienen una contenido mayor de 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, o 99% de, ya sea, cromosomas X o cromosomas Y.

Realizaciones de la invención pueden incluir espermatozoides recolectados de numerosas especies de

mamíferos macho, y la invención debería entenderse como que no está limitada a las especies de mamíferos machos descritas por los ejemplos específicos dentro de esta solicitud. En lugar de esto, los ejemplos específicos dentro de esta solicitud tienen por objeto ser ilustrativos de las variadas y numerosas especies de mamíferos machos a partir de los cuales se puede recolectar semen y este semen puede ser utilizado en ciertas realizaciones de la invención. Las realizaciones de la invención, por ejemplo, pueden incluir los espermatozoides de animales que tienen valor comercial para la producción de carne o producción láctea, tal como cerdos, ovino, bovinos, equinos, búfalos, o lo similar (naturalmente los mamíferos utilizados para la producción de carne o lácteo puede variar de cultura en cultura). Se puede también incluir los espermatozoides de diversas especies mamíferas domesticadas dentro de las que se encuentran los canes y los felino. También se pueden incluir espermatozoides de individuos de diversas especies mamíferas que tengan un atributo o atributos no comunes, tales como características morfológicas que incluyen peso, tamaño o conformación, u otras características deseadas tales como velocidad, agilidad, intelecto o lo similar. También se pueden incluir espermatozoides de primates, incluyendo, aunque no debe limitarse a éstos, chimpancés, gorilas o humanos y los espermatozoides de mamíferos marinos tales como ballenas y delfines. También se pueden incluir espermatozoides congelados-descongelados provenientes de todas las variedades de mamíferos anteriormente descritas y además, incluyendo aunque sin estar limitado a éstos, los espermatozoides de donadores fallecidos, de mamíferos raros o exóticos, especímenes zoológicos, o especies en peligro.

Haciendo ahora referencia principalmente a la figura 1, realizaciones particulares de la invención pueden comprender espermatozoides 1 que contienen semen recolectado a partir de un mamífero macho, incluyendo aunque no se debe limitar a éstos, aquellos anteriormente descritos. Los espermatozoides pueden ser incubados en una concentración de tintura 2, Hoechst 33342, de más de alrededor de 40 IM a una temperatura de entre alrededor de 30° Centígrados y alrededor de 40° Centígrados para una duración de tiempo de entre 50 minutos a 200 minutos para teñir ADN de espermatozoides con uniformidad suficiente para permitir que los espermatozoides que contienen cromosomas X se vean diferenciados de los espermatozoides que contienen cromosomas Y, en base a la magnitud de la fluorescencia en un rango mayor de alrededor del 85%.

La concentración de la tintura Hoechst 33342 entre 40 IM y 2500IM, la temperatura entre 30° Centígrados y alrededor de 40° Centígrados, y la duración de tiempo entre 50 minutos y 200 minutos puede seleccionarse para ajustar la pureza de las poblaciones separadas que contienen cromosomas X y cromosomas Y, o pueden seleccionarse para promover rangos de segmentación y desarrollo embrionario, tal como se describirá adicionalmente más adelante.

Por ejemplo, cuando se tiñe ADN de espermatozoides provenientes de ciertas especies bobinas, la concentración de la tintura Hoechst 33342 puede aumentarse hasta un valor de entre alrededor de 200IM y alrededor de 2500IM, se pueden incubar durante un período de tiempo de entre alrededor de 60 minutos a alrededor de 190 minutos a una temperatura de alrededor de 37° Centígrados. Específicamente con respecto a ciertos espermatozoides bovinos, congelados-descongelados, la tintura 2 Hoechst 33342 se puede ajustar para establecer una

concentración de 2240IM y luego se incuba durante alrededor de 60 minutos a alrededor de 39° Centígrados.

Con respecto a los rangos de segmentación de los oocitos inseminados con células de esperma mamífero de acuerdo con la invención, el incremento en la concentración de colorante hasta al menos 2240IM no parece tener un efecto depresivo ya sea en la segmentación o en el desarrollo embrionario. Concentraciones de colorantes más altas pueden realmente ser beneficiosas con respecto a ciertas realizaciones de la invención porque el tiempo de incubación puede disminuirse mejorando el porcentaje de segmentación o formación de blastocistos. De aplicación a aplicación la concentración de la tintura Hoechst 33342, la cantidad de tiempo de incubación, o ambos, se puede ajustar para obtener el máximo rango de segmentación y la máxima formación de blastocistos, si esto se desea.

Haciendo ahora referencia principalmente a las figuras 2 y 3, las realizaciones citométricas de flujo de acuerdo con la invención pueden incluir una fuente de células 3 que actúa para establecer o suministrar espermatozoides coloreados (frescos, congelados-descongelados, núcleos de esperma, o lo similar) para ser analizados por citometría de flujo. Las células son depositadas dentro de una boquilla o tobera 4 en una forma tal que las células de esperma teñidas son rodeadas por un fluido de cobertura o de envainado 5. El fluido de envainado 5 es suministrado usualmente por una fuente 6 de fluido de envainado de manera tal que mientras la fuente de células 3 suministra células de esperma, el fluido de envainado 5 es corrientemente alimentado a través de la boquilla 4. De esta manera el fluido de envainado 5 forma un entorno de fluido de envainado para las células de esperma. Dado que los diversos fluidos son provistos al citómetro de

flujo con alguna presión, los mismos fluyen hacia fuera de la boquilla 4 y salen por el orificio 7 de la boquilla. Mediante la previsión de un tipo de oscilador 8 que puede ser controlado muy precisamente a través de un control oscilador 9 las ondas de presión pueden ser establecidas dentro de la boquilla 4 y transmitidas a los fluidos que salen de la boquilla 4 por el orificio 7 de la boquilla. Dado que el oscilador 9 actúa en el fluido de envainado 5, la corriente 10 que sale del orificio de boquilla 7 eventualmente y regularmente forma gotas 11. Debido a que las células de esperma están por lo menos parcialmente rodeadas por un entorno de fluido de envainado, las gotas 11 pueden contener dentro de las mismas células de esperma individualmente aisladas.

Dado que las gotas 11 generalmente contienen células de esperma individualmente aisladas, el citómetro de flujo puede distinguir y separar pequeñas gotas en base a la magnitud de la fluorescencia emitida desde el fluorocromo adherido al ADN del los espermatozoides. Esto se consigue a través de una disposición 12 de sensado de células. La disposición de sensado de células comprende por lo menos algún tipo de sensor 13 que responde a la magnitud de la fluorescencia emitida por cada celular de esperma contenida dentro de cada gota 11. La disposición de sensado de células de esperma 13 puede provocar una acción dependiendo de la presencia relativa o de la ausencia relativa de fluorescencia emitida por el fluorocromo adherido ante la excitación provocada por algún estimulante tal como el excitador láser 14. Mientras cada espermatozoide puede ser teñido por fluorocromo, tal como la tintura Hoechst 33342, como se ha descrito anteriormente, la diferente cantidad de ADN que comprende el cromosoma X y el cromosoma Y provoca diferentes

cantidades de tintura que se adhiere. De esta manera, determinando mediante sensado el grado de fluorescencia emitida por el fluorocromo ante la excitación es posible discriminar entre espermatozoides que contienen cromosomas X y espermatozoides que contienen cromosomas Y por medio de sus diferentes niveles de emisión.

A los fines de conseguir la separación y la aislamiento de las apropiadas células de esperma, la señal es recibida por el sensor 14 son alimentadas son alimentadas a algún tipo de disposición 15 de discriminación y clasificación que muy rápidamente efectúa una decisión de diferenciación y puede cargar diferencialmente cada gota 11 en base a si ha decidido que la deseada célula de esperma existe o no existe dentro de aquella gota 11. De esta manera la disposición 15 de separación o discriminación actúa para permitir que las placas de deflexión electrostática 16 deflequen las gotas 11 basadas en si o no las mismas contienen la apropiada célula de esperma. Como resultado de esto, el citómetro de flujo actúa para seleccionar las células haciendo que las mismas caigan en uno o mas recolectores o elementos de contención 17. De esta manera, mediante el sensado de alguna propiedad de la célula de esperma (tal como la magnitud de la fluorescencia), el citómetro de flujo puede discriminar entre células de esperma en base aquella característica particular y ponerlas en el recolector o elemento de contención 17 apropiado. En realizaciones particulares de la invención que utilizan citometría de flujo para seleccionar espermatozoides, las gotas que contienen células de esperma que tiene cromosoma X son cargadas positivamente y de esta manera son desviadas en una dirección, y las gotas que contienen una célula de esperma que contiene cromosoma Y son cargadas negativamente y

de esta manera son desviadas en la otra dirección, y la corriente de deshecho (que contiene células de esperma que no se seleccionan) permanece no cargada y de esta manera puede ser recolectada en una corriente no desviada, dentro de un tubo de succión o lo similar.

Haciendo ahora referencia principalmente a la Figura 3, la boquilla 4 emite una corriente 10 que debido al oscilador 8 (no ilustrado en la Figura 3) forma gotas 11. Dado que la fuente 3 de células de esperma (no ilustrado en la Figura 3) puede suministrar células de esperma 1 que pueden estar teñidas de acuerdo con la invención anteriormente descrita, la emisión de luz desde el fotocromo adherido que es excitado por el excitador láser 13 puede ser determinada diferencialmente por medio del censor 14 de manera tal que la existencia o no existencia de una carga en cada gota 11 cuando esta se separa de la corriente 10 puede ser controlada por el citómetro de flujo. Este control da como resultado gotas 8 positivamente cargadas, negativamente cargadas o sin carga, basadas en la célula de esperma contenida dentro de cada gota 11. Tal como se muestra por medio de la Figura 3, se ilustran ciertas gotas como gotas desviadas 18. Estas gotas deflectadas o desviadas 18 son aquellas que contienen espermatozoides diferenciados por contener ya sea un cromosoma X o un cromosoma Y. El espermatozoide separado es luego aislado en un apropiado elemento de recolección o elemento de contención 17 para su uso posterior.

Realizaciones de la invención pueden comprender pequeñas gotas 11 cada una de las cuales contiene una célula de esperma 15 que contiene ya sea un cromosoma X o un cromosoma Y. Las gotas que contienen células de esperma que contienen cromosomas X pueden ser aisladas en un elemento o elementos de contención 17 a una velocidad o rango de por lo

menos 1000 por segundo o a un rango mayor de alrededor de 1000 por segundo tal como 2000 por segundo, 3000 por segundo, 4000 por segundo, 5000 por segundo, o mayor. De manera similar las células de esperma que contienen cromosomas Y pueden ser aisladas a una velocidad o rango de por lo menos 1000 por segundo, o a un rango mayor de alrededor de 1000 por segundo, tal como 2000 por segundo, 3000 por segundo, 4000 por segundo, 5000 por segundo o mayor. En algunas realizaciones de la invención, las gotas que contienen células de esperma que contienen cromosomas X y las gotas que contienen células de esperma que contienen cromosomas Y son simultáneamente separadas y aisladas en elementos de contención cada una a un rango de por lo menos 1000 por segundo, o mayor de 1000 por segundo, tal como 2000 por segundo, 3000 por segundo, 4000 por segundo, 5000 por segundo o aun en rangos mayores.

Realizaciones de la invención también pueden incluir muestras de inseminación artificial preparadas a partir de células de esperma recolectadas de mamíferos machos (que pueden estar congeladas y descongeladas con respecto a algunas realizaciones de la invención) que son luego teñidas y separadas de acuerdo con las realizaciones de la invención anteriormente descriptas. Las muestras de inseminación artificial pueden luego ser utilizadas en protocolos de inseminación artificial, por ejemplo, una muestra de inseminación artificial de un bovino preparada a partir de espermatozoides separados de acuerdo con la invención puede comprender menos de 10×10^6 de espermatozoides viables contenidos dentro de una cánula. Las muestras de inseminación artificial de baja dosis para inseminación artificial bovina pueden contener una cantidad tan baja como $1-3 \times 10^6$ de espermatozoides viables, o aún tan baja como 150.000

espermatozoides, tal como se describe en la solicitud de Patente de los Estados Unidos de Norteamérica N° 09/001.394, o solicitud de Patente PCT US98/27909, cada una de las cuales se incorpora a la presente a modo de referencia. Las muestras de inseminación artificial, que tienen un número regular de células de esperma separadas o una baja dosis de células de esperma separadas, pueden ser utilizadas en programas de cría de animales, tales como aquellos descritos en las solicitudes de Patente de los Estados Unidos de Norteamérica N° 60/224.050 y 60/21.093, cada una de las cuales se incorpora a la presente a modo de referencia. Las muestras de inseminación artificial que contienen espermatozoides previamente congelados y descongelados, teñidos y separados de acuerdo con la invención también pueden ser utilizadas en conjunción con programas sincronizados de cría que utilizan animales super-ovulados tal como se describe en la solicitud de Patente de los Estados Unidos de Norteamérica N° 09/001.454, que se incorpora a la presente a modo de referencia. Naturalmente, para células de esperma congeladas que tienen una limitada disponibilidad debido a que el mamífero macho ha muerto, o el mamífero macho es un animal raro o exótico, una muestra de inseminación artificial preparada de acuerdo con la invención puede contener aún un menor número de espermatozoides.

El número de espermatozoides separados viables que es teñido, separado, y aislado en poblaciones que contienen cromosomas X y poblaciones que contienen cromosomas Y de acuerdo con la invención, que son utilizados en una muestra de inseminación artificial, puede variar en base a las especies de mamíferos que serán inseminadas artificialmente. Por ejemplo, las muestras de inseminación artificial equinas preparadas a partir de espermatozoides separados pueden

requerir un mayor numero de espermatozoides viables separados con respecto a la aplicación bovina, tal como se describe en la solicitud de Patente PCT US99/17165 que se incorpora a la presenta a modo de referencia. Una realización de una muestra de inseminación equina puede, a modo de ejemplo, contener entre alrededor de 40 millones a alrededor de 100 millones de espermatozoides.

En ciertas realizaciones de la invención, la muestra de inseminación que contiene espermatozoides separados recolectados de un mamífero macho u obtenidos de esperma congelado-descongelado puede ser envasada para su uso con procedimientos de inseminación quirúrgica.

Las células de esperma teñidas, separadas o aisladas de acuerdo con la invención también pueden ser utilizadas para fertilizar un oocito u oocitos in-vitro (IVF). Una característica atractiva de la fertilización in-vitro (IVF) puede ser que sea la necesidad de menos esperma separado que lo que requiere la inseminación artificial. Puede ser deseable utilizar la menor cantidad de esperma posible, especialmente si el mamífero macho ha fallecido, es un mamífero raro o exótico o si el espermatozoide esta teñido o separado de acuerdo con diversas realizaciones de la invención. También, la disponibilidad comercial de las células de esperma separadas en poblaciones que contienen cromosomas Y, especialmente cuando el mamífero macho está ubicado a una distancia del mamífero hembra, o cuando el mamífero macho es exótico o raro o tiene atributos deseables, resultará probablemente en un mayor y más expandido uso de fertilización in-vitro en programas de cría. Ciertas realizaciones de la invención pueden incluir dispositivos y metodologías para el uso de espermatozoides separados, que incluyen, aunque no están limitados a éstos, a células de

esperma congeladas-descongeladas, con respecto a la fertilización in-vitro de oocitos, la maduración in-vitro de oocitos, o el cultivo in-vitro de cigotas, tal como aquellas descritas en el artículo publicado por Lu, K.H., Cran D.G., y Seidel, G.E., denominada fertilización in-vitro con esperma bovino clasificado por citometría de flujo, Theriogenology, 52, 1393-1405 (1999), que se incorpora a la presente a modo de referencia.

Ciertas realizaciones de la invención comprende la producción o generación de embriones mamíferos que pueden comprender la recolección de semen 1 a partir de un mamífero macho o la obtención de semen o espermatozoides 1 que están congelados o han sido previamente congelados. De acuerdo con las realizaciones de la invención descritas anteriormente, el semen es combinado con la tintura 2 Hoechst 33342 para establecer una concentración de entre 40 IM y 2500 IM. Las células de esperma son incubadas con la tintura Hoechst 33342 a una temperatura de entre alrededor de 30° Centígrados y alrededor de 40° Centígrados durante un tiempo de alrededor de 50 minutos a alrededor de 200 minutos. Las células de esperma teñidas pueden ser separadas y aisladas en poblaciones que contienen cromosomas X y poblaciones que contienen cromosomas Y de acuerdo con las realizaciones de la invención que se han descrito anteriormente o por medio de otras técnicas de separación de células de esperma que también pueden diferenciar los espermatozoides que contienen cromosomas X de aquellos espermatozoides que contienen cromosomas Y en base a la magnitud de la fluorescencia. Las células de esperma aisladas pueden luego ser utilizadas para fertilizar oocitos de un mamífero hembra de la misma especie, y en algunos casos de mamíferos hembra de diferentes especies, in-vitro.

Como ejemplo de una aplicación de realizaciones de la invención que comprenden esperma congelado de toro en aplicaciones de fertilización in-vitro IVF, se tiñieron muestras de esperma de dos toros en concentraciones de M o 2240M con tintura Hoechst 33342 y los espermatozoides teñidos fueron luego clasificados a granel en un citómetro de flujo a una velocidad de 1000 esperma por segundo en un citrato de yema de huevo al 2%. Los espermatozoides fueron inseminados a $1 \times 10^6/\text{mL}$ y los embriones fueron cultivados en el sistema mSOF descrito por Tervit H.R. y colaboradores, en Exitoso Cultivo In-Vitro de óvulos de oveja y ganado y J. Reprod. Fértil., 30:493-497(1992), que se incorpora a la presente a modo de referencia. Se llevaron a cabo tres replicados para un toro 1 y un replicado para un toro 2 (ver Tabla 1).

Tabla 1. Efecto de la concentración de tintura en los rangos de desarrollo y segmentación de oocitos inseminados con espermatozoides teñidos y separados a partir de esperma congelado-descongelado.

Toro	Nº de Eyaculaciones	Concent. (M) Hoechst 33342	Tiempo de tinción requerido (min)	Nº de oocitos	Segmentación % c	Blastocitos/Oocitos %
1	3	224	190	368	44 ^a	17
1	3	2240	60	373	60 ^b	23
2	1	224	190	86	23 ^a	0 ^a
2	1	2240	60	81	42 ^b	16 ^b

^{a,b} Porcentajes dentro de los toros dentro de las columnas con diferente superíndice difieren en ($P<.025$, ²).

Tal como se puede entender, puede tomar más tiempo teñir el esperma congelado-descongelado de manera tal que esto se pueda resolver durante la separación a una

concentración de tintura más baja que a una concentración de tintura 10X. Las diferencias observadas en los rangos de segmentación entre las dos concentraciones de tintura pueden ser más probablemente atribuidas al tiempo extendido de incubación al nivel de tintura inferior. Parecería que un aumento de 10 veces la concentración de tintura no tiene un efecto depresivo ni en la segmentación ni en el desarrollo embrionario.

Tal como se puede entender fácilmente de lo anterior, los conceptos básicos de la presente invención pueden ser llevados a cabo en una variedad de formas. Esto comprende el teñido de espermatozoides, ya sea espermatozoides frescos o espermatozoides congelados-descongelados, las técnicas de separación y aislamiento que pueden ser utilizadas con dichos espermatozoides congelados, así como también los dispositivos para llevar a cabo la tinción, separación y aislamiento de tales espermatozoides teñidos, que se separan en poblaciones que contienen cromosomas X y poblaciones que contienen cromosomas Y. En esta solicitud de patente, las técnicas de tinción y separación utilizadas con espermatozoides se describen como parte de los resultados que se muestran a ser logrados por los diversos dispositivos descritos y como etapas que son inherentes a la utilización. Los mismos son simplemente el resultado natural de utilizar los dispositivos tal como han sido descritos y definidos sus pósitos. Además, si bien se describen algunos dispositivos, debe entenderse que estos no solo llevan a cabo ciertos métodos sino que también pueden variarse en un número de formas. De manera importante, con referencia a todo lo anterior, la totalidad de estas facetas deben entenderse como que están comprendidas por ésta descripción.

La descripción incluida en ésta solicitud de patente internacional bajo el Tratado de Cooperación de Patentes tiene por objeto servir como una descripción básica. El lector debería advertir que la descripción específica puede no describir explícitamente toda las realizaciones posibles y que muchas alternativas son implícitas. Esta también puede no explicar completamente la naturaleza genérica de la invención y puede no mostrar explícitamente como cada característica o elemento puede ser realmente representativo de una función más amplia o de una gran variedad de elementos equivalentes o alternativos. Nuevamente, estos están implícitamente incluidos en ésta descripción. Cuando la invención es descrita en una terminología orientada a la funcionalidad cada aspecto de la función está conseguido por un dispositivo, su rutina o programa. Las reivindicaciones de aparato pueden no solamente estar incluidas para los dispositivos descritos, sino que también las reivindicaciones de método o proceso pueden estar incluidas para encargarse de las funciones de la invención y de la que cada elemento lleva a cabo. Ni la descripción ni la terminología tienen por objeto limitar el alcance de las reivindicaciones que ahora se incluyen.

Además, cada uno de los diversos elementos de la invención y las reivindicaciones, puede también ser conseguido en una variedad de formas. Esta descripción deberá entenderse como que comprende cada una de tales variaciones ya sea una variación de una realización de cualquier realización de aparato, de método o de proceso o aun meramente una variación de cualquier elemento de estos. Particularmente, deberá entenderse que dado que la descripción se refiere a elementos de la invención, las palabras para cada elemento pueden ser expresadas por medio

de términos equivalentes de aparato o términos de método, aun si solamente la función o el resultado es el mismo. Tales términos equivalentes, mas amplios, o aun mas genéricos deberían ser considerados como comprendidos en la descripción de cada elemento o acción. Tales términos pueden ser sustituidos cuando se desee para hacer explicita la cobertura implícitamente amplia que tiene por objeto la presente invención. A modo de un ejemplo, deberá entenderse que todas las acciones pueden ser expresadas como un medio para tomar dicha acción o como un elemento que provoca dicha acción. De manera similar cada elemento físico descrito debería entenderse como que comprende una descripción de la acción que dicho elemento físico facilita. Con respecto a este ultimo aspecto, a modo de ejemplo, la descripción de un "clasificador" debería entenderse como que comprende la descripción del acto de "clasificación" ya sea que esta explícitamente descrito o no y, inversamente, cuando se brinde solamente la descripción del acto de "clasificación", dicha descripción deberá entenderse como que comprende la descripción de un "clasificador" y aun un "medio para clasificar o seleccionar". Tales cambios y términos alternativos deben entenderse como que están explícitamente incluidos en la descripción. De manera adicional, las diversas combinaciones y permutaciones de todos los elementos o aplicaciones pueden ser creadas y presentadas. Todo puede hacerse para optimizar el diseño o comportamiento en una aplicación específica.

Cualquier acto de la ley, estatuto, regulación, o regla mencionada en esta solicitud de patente, o patentes, publicaciones u otras referencias mencionadas en esta solicitud de patente se incorporan a modo de referencia. Específicamente, la solicitud de patente provisional de los

Estados Unidos de Norteamérica N 60/253.787, presentada el 29 de noviembre de 2000, y la solicitud de patente provisional de los Estados Unidos de Norteamérica N° 60/253.785, presentada el 29 de noviembre de 2000, se incorporan a la presente a modo de referencia, incluyendo cualquier figura o adjunto y cada una de las referencias en la tabla de referencias siguiente se incorpora a la presente a modo de referencia.

La bibliografía que se menciona en la presente será respetada en su idioma original para facilitar la identificación de la misma.

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Además, con referencia a cada término utilizado deberá comprenderse que a menos que su utilización en esta solicitud de patente sea inconsistente con su interpretación, las definiciones comunes de diccionario deberán entenderse como incorporadas para cada término y todas las definiciones, términos alternativos y sinónimos tales como los contenidos en el diccionario Random House Webmaster's Unabridged, segunda edición, que se incorpora a la presente a modo de referencia. Sin embargo, con respecto a lo anterior, en el caso que dicha información o declaraciones incorporadas a modo de referencia pudieran ser consideradas inconsistentes con el patentamiento de esta invención o invenciones tales declaraciones expresamente no deben ser consideradas como hechas por el solicitante o los solicitantes.

Además, a menos que el contexto lo requiera de otro modo, deberá entenderse que el término "comprende" o variaciones tales como "comprenden" "que comprende" o "comprendiendo" tiene por objeto implicar la inclusión de un elemento o etapa o grupo de elementos o etapas mencionados pero no la exclusión de ningún otro elemento o etapa o grupo de elementos o etapas. Tales términos deben ser interpretados en su forma mas expansiva de manera de proveerle al solicitante la mas amplia cobertura legalmente permitida en países tales como Australia o lo similar.

De esta manera el solicitante o los solicitantes deberá entenderse como que cuentan con el apoyo como para reivindicar por lo menos: i) cada uno de los procedimientos de tinción o coloración, separación, aislamiento, inseminación, o fertilización tal como aquí se describen y explican, ii) los métodos relacionados descriptos y explicados, iii) variaciones similares, equivalentes y aun implícitas de cada uno de estos dispositivos y métodos, iv) aquellos diseños alternativos que consiguen cada una de las funciones que se ilustran como que están descriptas y explicadas, v) aquellos diseños alternativos y métodos alternativos que consiguen cada una de las funciones ilustradas, como implícitas para conseguir aquella que esta descrita y explicada, vi) cada característica componente y etapa ilustrada, como invenciones separadas en independientes, vii) las aplicaciones mejoradas por las diversas disposiciones o componentes descriptos, viii) los productos resultantes producidos por tales disposiciones o componentes, ix) métodos y aparatos sustancialmente tales como se han descrito hasta aquí y con referencia a cualquiera de los elementos que se acompañan, y x) las diversas y variadas combinaciones y permutaciones de cada uno de los elementos descritos.

Las reivindicaciones tales como se definen en esta solicitud están aquí incorporadas a modo de referencia como parte de esta descripción de la invención y el solicitante expresamente se reserva el derecho de usar la totalidad o una porción de dicho contenido incorporado de tales reivindicaciones como descripción adicional para soportar cualquier o la totalidad de las reivindicaciones o cualquier elemento o componente de las mismas, y el solicitante además se reserva expresamente el derecho de mover cualquier porción

de, o la totalidad de, el contenido incorporado de tales reivindicaciones o cualquier elemento componente de las mismas, desde la descripción hasta las reivindicaciones o viceversa en medida que sea necesario para definir el objeto por el cual se pretende protección por medio de esta solicitud o por cualquier solicitud subsiguiente de continuación, división o solicitud de mejor con respecto a la misma, o para obtener cualquier beneficio, reducción de tasas, en base a o para cumplir con las leyes, reglas o regulaciones de patentes de cualquier país o tratado y dicho contenido incorporado a modo de referencia permanecerá vigente durante la totalidad de la vigencia de esta solicitud incluyendo cualquier solicitud subsiguiente de continuación, división, o solicitud adicional o de mejora referida a la misma o cualquier extensión o re-concesión de la misma.

REIVINDICACIONES

Habiendo así especialmente descrito y determinado la naturaleza de la presente invención y la forma como la misma ha de ser llevada a la práctica, se declara reivindicar como de propiedad y derecho exclusivo:

1. Un método para la tinción de células de esperma recolectada de mamíferos, que comprende las etapas de:

a. recolectar semen de un mamífero macho;

b. incubar células de esperma contenidas dentro de dicho semen en una concentración micro-molar de colorante Hoechst 33342 mayor de 40;

c. establecer la temperatura a la cual dichas células de esperma en dicha concentración de colorante Hoechst 33342 son incubadas entre alrededor de 30°C y alrededor de 40°C;

d. ajustar un tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 entre alrededor de 50 minutos y alrededor de 200 minutos; y

e. teñir el ADN dentro de dichas células de esperma con una uniformidad suficiente para permitir que las células de esperma que contienen cromosomas X sean diferenciadas de las células de esperma que contienen cromosomas Y en base a la magnitud de fluorescencia en un rango mayor de alrededor de 85%.

2. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 1, donde dicho mamífero macho está seleccionado del grupo de mamíferos que consiste de primates,

humanos, porcinos, ovinos, bóvidos, equinos, cánidos, felinos y delfines.

3. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 2, donde dicho mamífero macho comprende dicho bóvido y dicha concentración micro-molar de colorante Hoechst 33342 es de entre alrededor de 200 y alrededor de 2500.

4. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 2, donde dicho mamífero macho comprende dicho bóvido y dicha concentración micro-molar de colorante Hoechst 33342 es de 224.

5. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 2, donde dicho mamífero macho comprende dicho bóvido y dicha concentración micro-molar de colorante Hoechst 33342 es de 2240.

6. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 4, donde dicha etapa de ajustar un tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 entre alrededor de 50 minutos y alrededor de 200 minutos comprende ajustar dicho tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 a alrededor de 190 minutos.

7. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 5, donde dicha etapa de ajustar un tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 entre alrededor de 50 minutos y alrededor de 200 minutos comprende ajustar dicho tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 a alrededor de 60 minutos.

8. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 1, donde dicha etapa de teñir el ADN dentro de dichas células de esperma con una uniformidad suficiente para permitir que las células de esperma que contienen cromosomas X sean diferenciadas de las células de esperma que contienen cromosomas Y en base a la magnitud de fluorescencia en un rango mayor de alrededor de 85% comprende diferenciar las células de esperma que contienen cromosomas Y de las células de esperma que contienen cromosomas X en base a la magnitud de fluorescencia a un rango seleccionado del grupo consistente en el 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% o 99%.

9. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 8, donde dichas células de esperma que contienen cromosomas X diferenciadas de las células de esperma que contienen cromosomas Y comprenden células de esperma viables.

10. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en las reivindicaciones 1, 2, 6, 7, 8 o 9, que comprende además la etapa de congelar dicho semen.

11. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 10, que comprende además la etapa de descongelar dicho semen.

12. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 11, donde dicha etapa de teñir el ADN dentro de dichas células de esperma con una uniformidad suficiente para permitir que las células de esperma que contienen cromosomas X sean diferenciadas de las células de esperma que contienen cromosomas Y en base a la magnitud de fluorescencia en un rango mayor de alrededor de 85% comprende diferenciar dicha magnitud de fluorescencia con un citómetro de flujo.

13. Un método para la tinción de ADN dentro de células de esperma congelado-descongelado, que comprende las etapas de:

a. recolectar semen que contiene células de esperma de un mamífero macho;

b. congelar dicho semen que contiene dichas células de esperma;

c. descongelar dicho semen que contiene dichas células de esperma;

d. combinar semen congelado-descongelado con colorante Hoechst 33342;

e. establecer una concentración micro-molar de dicho colorante Hoechst 33342 de entre alrededor de 200 y alrededor de 2500;

f. ajustar la temperatura de dicho semen congelado-descongelado en dicha concentración de colorante Hoechst 33342 entre alrededor de 30°C y alrededor de 40°C; y

g. ajustar el tiempo de duración en que dicho semen congelado-descongelado es incubado con dicha concentración de colorante Hoechst 33342 entre alrededor de 50 minutos y alrededor de 200 minutos, de modo que el ADN dentro de las células de esperma contenidas en dicho semen congelado-descongelado es teñido con una uniformidad suficiente para diferenciar entre las células de esperma que contienen cromosomas X y las células de esperma que contienen cromosomas Y en base a la magnitud de fluorescencia.

14. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 13, donde dicho mamífero macho está seleccionado del grupo de mamíferos que consiste de primates, humanos, porcinos, ovinos, bóvidos, equinos, cánidos, felinos y delfines.

15. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 14, donde dicho mamífero macho comprende dicho bóvido y dicha concentración micro-molar de colorante Hoechst 33342 es de entre alrededor de 200 y alrededor de 2500.

16. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 14, donde dicho mamífero macho comprende dicho

bóvido y dicha concentración micro-molar de colorante Hoechst 33342 es de 224.

17. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 14, donde dicho mamífero macho comprende dicho bóvido y dicha concentración micro-molar de colorante Hoechst 33342 es de 2240.

18. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 14, donde dicha etapa de ajustar un tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 entre alrededor de 50 minutos y alrededor de 200 minutos comprende ajustar dicho tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 a alrededor de 190 minutos.

19. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 17, donde dicha etapa de ajustar un tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 entre alrededor de 50 minutos y alrededor de 200 minutos comprende ajustar dicho tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 a alrededor de 60 minutos.

20. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en las reivindicaciones 13, 15, 16, 17, 18, 19 o 20, donde dicha

etapa de teñir el ADN dentro de dichas células de esperma con una uniformidad suficiente para permitir que las células de esperma que contienen cromosomas X sean diferenciadas de las células de esperma que contienen cromosomas Y en base a la magnitud de fluorescencia en un rango mayor de alrededor de 85% comprende diferenciar las células de esperma que contienen cromosomas Y de las células de esperma que contienen cromosomas X en base a la magnitud de fluorescencia a un rango seleccionado del grupo consistente en el 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% o 99%.

21. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 20, donde dichas células de esperma que contienen cromosomas X diferenciadas de las células de esperma que contienen cromosomas Y comprenden células de esperma viables.

22. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 20, donde dicha etapa de teñir el ADN dentro de dichas células de esperma con una uniformidad suficiente para permitir que las células de esperma que contienen cromosomas X sean diferenciadas de las células de esperma que contienen cromosomas Y en base a la magnitud de fluorescencia en un rango mayor de alrededor de 85% comprende diferenciar dicha magnitud de fluorescencia con un citómetro de flujo.

23. Un método para generar embriones de mamífero, que comprende las etapas de:

- a. recolectar semen de un mamífero macho;

b. combinar dicho semen de un mamífero macho con una cantidad de colorante Hoechst 33342;

c. establecer una concentración de dicho colorante Hoechst 33342 combinado con dicho semen a una concentración micro-molar de entre alrededor de 40 y alrededor de 2500;

d. ajustar la temperatura a la cual dichas células de esperma son incubadas con dicho colorante Hoechst 33342 entre alrededor de 30°C y alrededor de 40°C;

e. ajustar el tiempo de duración en que dichas células de esperma son incubadas en dicha concentración de colorante Hoechst 33342, entre alrededor de 60 minutos y alrededor de 200 minutos;

f. teñir el ADN dentro de las células de esperma contenidas en dicho semen con dicho colorante Hoechst 33342; y

g. fertilizar oocitos con células de esperma teñido, de modo que el aumento de la concentración de colorante Hoechst 33342 y la disminución del tiempo de duración en que dichas células de esperma son incubadas en dicha concentración de colorante Hoechst 33342, aumenta el porcentaje de dichos embriones de mamífero producidos.

24. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 23, donde dicho mamífero macho está seleccionado del grupo de mamíferos que consiste de primates, humanos, porcinos, ovinos, bóvidos, equinos, cánidos, felinos y delfines.

25. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 24, donde dicho mamífero macho comprende dicho

bóvido y dicha concentración micro-molar de colorante Hoechst 33342 es de entre alrededor de 200 y alrededor de 2500.

26. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 24, donde dicho mamífero macho comprende dicho bóvido y dicha concentración micro-molar de colorante Hoechst 33342 es de 224.

27. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 24, donde dicho mamífero macho comprende dicho bóvido y dicha concentración micro-molar de colorante Hoechst 33342 es de 2240.

28. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 26, donde dicha etapa de ajustar un tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 entre alrededor de 50 minutos y alrededor de 200 minutos comprende ajustar dicho tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 a alrededor de 190 minutos.

29. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 27, donde dicha etapa de ajustar un tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 entre alrededor de 50 minutos y alrededor de 200 minutos comprende ajustar dicho tiempo de duración en que dichas células de

esperma son incubadas con dicha concentración de colorante Hoechst 33342 a alrededor de 60 minutos.

30. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en las reivindicaciones 23, 25, 26, 27, 28, 29 o 30, donde dicha etapa de teñir el ADN dentro de dichas células de esperma con una uniformidad suficiente para permitir que las células de esperma que contienen cromosomas X sean diferenciadas de las células de esperma que contienen cromosomas Y en base a la magnitud de fluorescencia en un rango mayor de alrededor de 85% comprende diferenciar las células de esperma que contienen cromosomas Y de las células de esperma que contienen cromosomas X en base a la magnitud de fluorescencia a un rango seleccionado del grupo consistente en el 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% o 99%.

31. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 30, donde dichas células de esperma que contienen cromosomas X diferenciadas de las células de esperma que contienen cromosomas Y comprenden células de esperma viables.

32. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 30, donde dicha etapa de teñir el ADN dentro de dichas células de esperma con una uniformidad suficiente para permitir que las células de esperma que contienen cromosomas X sean diferenciadas de las células de esperma que contienen cromosomas Y en base a la magnitud de fluorescencia

en un rango mayor de alrededor de 85% comprende diferenciar dicha magnitud de fluorescencia con un citómetro de flujo.

33. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 32, que comprende además la etapa de aislar las células de esperma diferenciadas, que contienen cromosomas X y que contienen cromosomas Y, en elementos de recolección separados.

34. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 33, donde dicha etapa de aislar las células de esperma diferenciadas, que contienen cromosomas X y que contienen cromosomas Y, en elementos recolectados separados comprende aislar las células de esperma que contienen cromosomas Y en un elemento de recolección separado a un rango de alrededor de 1000 por segundo.

35. Un método para la tinción de células de esperma recolectada de mamíferos, tal como se define en la reivindicación 33, donde dicha etapa de aislar las células de esperma diferenciadas, que contienen cromosomas X y que contienen cromosomas Y, en elementos recolectados separados comprende aislar las células de esperma que contienen cromosomas X en un elemento de recolección separado a un rango de alrededor de 1000 por segundo.

36. Un método para generar embriones de mamífero, tal como se define en la reivindicación 23, que comprende además la etapa de congelar dicho semen.

37. Un método para generar embriones de mamífero, tal como se define en la reivindicación 30, que comprende además la etapa de congelar dicho semen.

38. Un método para generar embriones de mamífero, tal como se define en la reivindicación 36, que comprende además la etapa de descongelar dicho semen.

39. Un método para generar embriones de mamífero, tal como se define en la reivindicación 37, que comprende además la etapa de descongelar dicho semen.

40. Una disposición de citómetro de flujo para aislar células de esperma deseadas, que comprende:

a. células de esperma obtenidas descongelando semen previamente congelado, en donde dichas células de esperma son incubadas con colorante Hoechst 33342 en una concentración micro-molar de entre alrededor de 200 y alrededor de 2500 hasta que el ADN dentro de dichas células de esperma se tñía con suficiente uniformidad como para permitir que las células de esperma que contienen cromosomas X sean diferenciadas de las células de esperma que contienen cromosomas Y en base a la magnitud de la fluorescencia en un rango mayor de alrededor de 85%;

b. una fuente de células de esperma que suministra dichas células de esperma a un citómetro de flujo;

c. una fuente de fluido de envainado que crea un entorno de fluido de envainado dentro de dicho citómetro de flujo en el cual dichas células de esperma están retenidas;

d. una boquilla a través de la cual pasan dichas células de esperma mientras están retenidas en dicho entorno de fluido de envainado;

e. un oscilador que actúa en dicho fluido de envainado cuando este pasa a través de dicha boquilla;

f. una disposición sensora de células de esperma que responde a dichas células de esperma;

g. una disposición de separación y discriminación que actúa para separar dichas células de esperma que tienen una característica deseada; y

h. un elemento de contención dentro de cual son recolectadas dichas células de esperma que tienen una característica deseada.

41. Una disposición de citómetro de flujo para aislar células de esperma deseadas, tal como se define en la reivindicación 40, donde dichas células de esperma obtenidas descongelando semen previamente congelado son obtenidas de mamíferos machos seleccionados del grupo de mamíferos que consiste de primates, humanos, porcinos, ovinos, bóvidos, equinos, cánidos, felinos y delfines.

42. Una disposición de citómetro de flujo para aislar células de esperma deseadas, tal como se define en la reivindicación 41, donde dicho mamífero macho comprende dicho bóvido y donde dicha concentración micro-molar de colorante Hoechst 33342 es de entre alrededor de 200 y alrededor de 2500.

43. Una disposición de citómetro de flujo para aislar células de esperma deseadas, tal como se define en la reivindicación 41, donde dicho mamífero macho comprende dicho bóvido y donde dicha concentración micro-molar de colorante Hoechst 33342 es de 224.

44. Una disposición de citómetro de flujo para aislar células de esperma deseadas, tal como se define en la reivindicación 41, donde dicho mamífero macho comprende dicho bóvido y donde dicha concentración micro-molar de colorante Hoechst 33342 es de 2240.

45. Una disposición de citómetro de flujo para aislar células de esperma deseadas, tal como se define en la reivindicación 42, que comprende además un tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 entre alrededor de 50 minutos y alrededor de 200 minutos.

46. Una disposición de citómetro de flujo para aislar células de esperma deseadas, tal como se define en la reivindicación 43, donde dicho tiempo de duración es de alrededor de 190 minutos.

47. Una disposición de citómetro de flujo para aislar células de esperma deseadas, tal como se define en la reivindicación 43, donde dicho tiempo de duración es de alrededor de 60 minutos.

48. Una disposición de citómetro de flujo para aislar células de esperma deseadas, tal como se define en las reivindicaciones 40, 42, 43, 45 ó 48, donde dichas células de esperma que contienen cromosomas X son diferenciadas de las células de esperma que contienen cromosomas Y en base a la magnitud de fluorescencia en un rango mayor de alrededor de 85%, que es un rango seleccionado del grupo consistente en el 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% o 99%.

49. Una disposición de citómetro de flujo para aislar células de esperma deseadas, tal como se define en la reivindicación 48, que comprende además un elemento de recolección dentro del cual están aisladas las células de esperma que contienen cromosomas X.

50. Una disposición de citómetro de flujo para aislar células de esperma deseadas, tal como se define en la reivindicación 48, que comprende además un elemento de recolección dentro del cual están aisladas las células de esperma que contienen cromosomas Y.

51. Una disposición de citómetro de flujo para aislar células de esperma deseadas, tal como se define en la reivindicación 49, que comprende además un rango al cual se aíslan las células de esperma que contienen cromosomas X mayor de alrededor de 1000 por segundo.

52. Una disposición de citómetro de flujo para aislar células de esperma deseadas, tal como se define en la reivindicación 50, que comprende además un rango al cual se aíslan las células de esperma que contienen cromosomas Y mayor de alrededor de 1000 por segundo.

53. Un método para generar embriones de mamífero, tal como se define en la reivindicación 40, que comprende además la etapa de congelar dicho semen.

54. Un método para generar embriones de mamífero, tal como se define en la reivindicación 51, que comprende además la etapa de congelar dicho semen.

55. Un método para generar embriones de mamífero, tal como se define en la reivindicación 53, que comprende además la etapa de descongelar dicho semen.

56. Un método para generar embriones de mamífero, tal como se define en la reivindicación 54, que comprende además la etapa de descongelar dicho semen.

57. Un método para producir un de mamífero que tiene un sexo predeterminado, que comprende las etapas de:

- a. recolectar semen de un mamífero macho;
- b. congelar dicho semen;
- c. descongelar dicho semen;
- d. determinar la característica del sexo de una pluralidad de células de esperma contenidas dentro de dicho semen congelado-descongelado;
- e. separar dichas células de esperma de acuerdo con la determinación de sus características de sexo;
- f. aislar células de esperma separadas de acuerdo con la determinación de sus características de sexo en un elemento recolector;
- g. establecer una muestra de inseminación artificial de dichas células de esperma aisladas en dicho elemento de recolección;
- h. insertar dicha muestra de inseminación artificial dentro de un mamífero hembra de la misma especie de la cual se ha recolectado dicho semen;
- f. fertilizar por lo menos un huevo dentro de dicho mamífero hembra; y
- g. producir un mamífero de prole del sexo deseado.

58. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la reivindicación 57, donde dicho mamífero macho está seleccionado del grupo de mamíferos que consiste de primates, humanos, porcinos, ovinos, bóvidos, equinos, cánidos, felinos y delfines.

59. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la reivindicación 58, que comprende además la etapa de teñir el ADN dentro de dichas células de esperma en una concentración micro-molar mayor de 40 con un colorante Hoechst 33342.

60. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la reivindicación 59, donde dicha etapa de teñir el ADN dentro de dichas células de esperma en una concentración micro-molar mayor de 40 con un colorante Hoechst 33342 comprende la tinción con una uniformidad suficiente para permitir que las células de esperma que contienen cromosomas X sean diferenciadas de las células de esperma que contienen cromosomas Y en base a la magnitud de fluorescencia en un rango mayor de alrededor de 85%.

61. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la reivindicación 59, donde dicho mamífero macho comprende dicho bóvido y dicha concentración micro-molar de colorante Hoechst 33342 es de entre alrededor de 200 y alrededor de 2500.

62. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la

reivindicación 61, donde dicho mamífero macho comprende dicho bóvido y dicha concentración micro-molar de colorante Hoechst 33342 es de 224.

63. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la reivindicación 61, donde dicho mamífero macho comprende dicho bóvido y dicha concentración micro-molar de colorante Hoechst 33342 es de 2240.

64. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la reivindicación 61, que comprende además la etapa de ajustar un tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 entre alrededor de 50 minutos y alrededor de 200 minutos.

65. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la reivindicación 61, donde dicha etapa de ajustar un tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 entre alrededor de 50 minutos y alrededor de 200 minutos comprende ajustar dicho tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 a alrededor de 190 minutos.

66. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la reivindicación 63, donde dicha etapa de ajustar un tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 entre

alrededor de 50 minutos y alrededor de 200 minutos comprende ajustar dicho tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 33342 a alrededor de 60 minutos.

67. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en las reivindicaciones 57, 58, 61, 62, 63, 64, 65 o 66, donde dicha etapa de teñir el ADN dentro de dichas células de esperma en una concentración micro-molar mayor de 40 con un colorante Hoechst 33342, comprende la tinción con una uniformidad suficiente para permitir que las células de esperma que contienen cromosomas X sean diferenciadas de las células de esperma que contienen cromosomas Y en base a la magnitud de fluorescencia en un rango mayor de alrededor de 85% y que comprende un rango seleccionado del grupo consistente en el 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% o 99%.

68. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la reivindicación 67, donde dicha etapa de teñir el ADN dentro de dichas células de esperma con una uniformidad suficiente para permitir que las células de esperma que contienen cromosomas X sean diferenciadas de las células de esperma que contienen cromosomas Y en base a la magnitud de fluorescencia en un rango mayor de alrededor de 85% comprende diferenciar dicha magnitud de fluorescencia con un citómetro de flujo.

69. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la reivindicación 68, donde dicha etapa de aislar las células de

esperma separadas de acuerdo con la determinación de su sexo en un elemento de recolección comprende aislar las células de esperma que contienen cromosomas Y en un elemento de recolección separado a un rango de alrededor de 1000 por segundo.

70. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la reivindicación 68, donde dicha etapa de aislar las células de esperma separadas de acuerdo con la determinación de su sexo en un elemento de recolección comprende aislar las células de esperma que contienen cromosomas X en un elemento de recolección separado a un rango de alrededor de 1000 por segundo.

71. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la reivindicación 57, que comprende además la etapa de limitar el número de células aisladas de esperma en dicha muestra de inseminación artificial entre alrededor del 10% y alrededor del 50% del número de dichas células de esperma con respecto a una típica muestra de inseminación artificial no separada.

72. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la reivindicación 58, donde dicho mamífero comprende dicho bóvido y donde dicha muestra de inseminación artificial tiene el número de células aisladas de esperma limitado a alrededor de un millón a tres millones.

73. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la

reivindicación 58, donde dicho mamífero comprende dicho bóvido y donde dicha muestra de inseminación artificial tiene el número de células aisladas de esperma limitado a entre alrededor de cien mil y cincuenta mil y alrededor de un millón.

74. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en la reivindicación 57, donde dicho mamífero comprende dicho equino y donde dicha muestra de inseminación artificial tiene el número de células aisladas de esperma limitado a entre alrededor de cuarenta millones y alrededor de cien millones.

75. Un método para producir un de mamífero que tiene un sexo predeterminado, tal como se define en las reivindicaciones 71, 72, 73 o 74, que comprende además la etapa de crear superovulación en dicho mamífero hembra para crear por lo menos dos huevos, que comprende la etapa de utilizar un producto farmacéutico ovulatorio para provocar la producción de múltiples huevos, y donde dicho producto farmacéutico ovulatorio es inyectado en incrementos de medio día entre cualquiera de los días 2 y 18 del ciclo de estro.

RESUMEN

Dispositivos, composiciones y métodos para manipular, separar, envasar y utilizar espermatozoides 1 que pueden ser derivados de muestras de esperma previamente congeladas y recolectadas de un mamífero macho. Específicamente, técnicas para teñir 2 uniformemente ADN de espermatozoides aun cuando éstos derivan de esperma previamente congelado y técnicas de separación para separar y aislar espermatozoides aun cuando éstos derivan de muestras de esperma previamente congelado, en poblaciones que contienen cromosomas X y cromosomas Y, y que tienen alta pureza.

Expediente No		030 548 28	No de Folio
Item		No de unidades	✓
1	CD		
2	DVD		
3	REVISTA		
4	LIBRO		
5	PERIÓDICO		
6	DISQUETES		
7	SOBRE DE MANILA X	1	✓
8	SOBRE BLANCO TAMAÑO CARTA		
9	SOBRE BLANCO TAMAÑO OFICIO		
10	OTROS:		

Observaciones:

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MIT : 800176089-2

SUPERINDUSTRIA Y COMERCIO

Radicacion : 03054620 00000000

Folios: 120

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Tramite : 011 PCT-PATENT 379 PAGE INCI 411 PRESENTAC

Dependencia: 8020 DIVISION DE NUEVAS CREACIONES

RECIBO OFICIAL DE CAJA : 03 - 44,015
FECHA : JUNIO 24 DE 2003

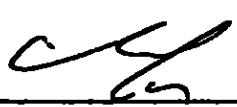
***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	Vr. PAGO
CAVELIER ABOGADOS	CONSIGNACION	BANCO POPULAR	050-00110-6	11076229	24/06/2003	26,973,000.00

***** CONCEPTO *****

CANT. HISTORICO	CONCEPTO	TOTAL CONCEPTO
1 50005-01-01 SOLICITUDES	1 TRAMITES DE SOL. DE PATENTE DE IN	400,000.00
	TOTAL :	\$ 400,000.00

SON: CUATROCIENTOS MIL PESOS

RESPONSABLE : 

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

Bo/o 13534-841-002-02

REQUISITOS NECESARIOS PARA PRESENTACION Y AGILIZACION DEL TRAMITE

	USUARIO	RADICADOR
PATENTE DE INVENCION	()	()
MODELO DE UTILIDAD	()	()
- Indicación de que se solicita la concesión de una patente.	()	()
- Datos de identificación del solicitante o de la persona que se presenta la solicitud.	()	()
- Descripción de la invención.	()	()
- Dibujos de ser estos pertinentes.	()	()
- Comprobante de Pago de las tasas establecidas.	()	()

COMPLETA () INCOMPLETA ()

DISEÑO INDUSTRIAL ()		
- Indicación de que se solicita el registro de Diseño Industrial	()	()
- Datos de identificación del solicitante o de la persona que presenta la solicitud.	()	()
- Representación gráfica y fotográfica del Diseño Industrial o muestra del Material que incorpora el diseño.	()	()
- Comprobante de pago de las tasas establecidas	()	()

COMPLETA () INCOMPLETA ()

ESQUEMA DE TRAZADO ()		
- Indicación de que solicita el registro de un Esquema de trazado.	()	()
- Datos de identificación del solicitante o de la persona que presenta La solicitud.	()	()
- Representación gráfica del esquema de trazado	()	()
- Comprobante de pago de las tasas establecidas.	()	()

COMPLETA () INCOMPLETA ()

Firma Responsable:



SUPERINDUSTRIA Y COMERCIO

Radicación : 83054128 00000000

Folios: 128

Fecha: Fecha (HDD): 2003-06-27 17:52:13

Trámite : 811 PCT-PTENT 379 FASE INCI 411 PRESENTAC

Dependencia: 2020 DIVISION DE NUEVAS CREACIONES



2020
Bogotá, D.C.,

Folio 122

Doctor:
Emilio Ferrero
Apoderado
XY, INC y Colorado State.

Oficio N° **07512**

ASUNTO:	Radicación N°	03-54828
	Solicitud Internacional	PCT/US2001/45023
	Fecha Inicio fase nacional	29/06/2003
	Trámite	011
	Evento	379
	Actuación	430
	Folios	001

Como resultado del examen de forma, realizado con fundamento en el artículo 38 de la Decisión 486 de la Comisión de la Comunidad Andina, artículo 27 del Tratado de Cooperación en Materia de Patentes (PCT), regla 51 bis del Reglamento y Circular Única de la Superintendencia de Industria y Comercio, se le comunica que:

- ☐ La solicitud no contiene la copia del documento en que consta la cesión del derecho a la patente del inventor al solicitante o a su causante. (Art. 26-k) Decisión 486).

En cumplimiento de lo dispuesto por el artículo 39 de la Decisión 486, se le requiere para que dentro del término de dos meses siguientes a la fecha de notificación del presente oficio, complete los requisitos anteriormente enunciados. En caso de no dar cumplimiento al presente requerimiento, la solicitud se considerará abandonada y perderá su prelación.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.


ALIX CARMENZA CESPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones.

17 SET. 2003

El anterior requerimiento fue notificado por fijación en lista de fecha _____.