



Señor

**SUPERINTENDENTE DE INDUSTRIA Y COMERCIO**

Superintendencia de Industria y Comercio

E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Referencia : Expediente No. 03-054828A  
Asunto : Solicitud de patente para **DISPOSICIÓN DE SEPARAR ESPERMATOZOIDES CONGELADOS EN POBLACIONES QUE CONTIENEN CROMOSOMAS X y CROMOSOMAS Y.**  
Solicitante : XY, LLC.

## **I. RECURSO VÍA GUBERNATIVA**

MARCELA ROJAS ROJAS, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, XY, LLC., por el presente escrito atentamente manifiesto a usted que interpongo recurso de **REPOSICIÓN** contra la Resolución No. 47353 del 31 de Agosto de 2011, para que sea revocada y en consecuencia se conceda el privilegio de patente para la invención **“DISPOSICION PARA SEPARAR ESPERMATOZOIDES CONGELADOS EN POBLACIONES QUE CONTIENEN CROMOSOMAS X Y CROMOSOMAS Y”**.

## **II. PETICIONES**

1. Se revoque la Resolución No. 47353 del 31 de Agosto de 2011 mediante la cual se deniega el privilegio de patente a la invención **“DISPOSICION PARA SEPARAR ESPERMATOZOIDES CONGELADOS EN POBLACIONES QUE CONTIENEN CROMOSOMAS X Y CROMOSOMAS Y”**;
2. Como consecuencia de la anterior declaración se proceda a conceder el privilegio de patente a la invención **“DISPOSICION PARA SEPARAR ESPERMATOZOIDES CONGELADOS EN POBLACIONES QUE CONTIENEN CROMOSOMAS X Y CROMOSOMAS Y”** y asignar el respectivo número de título de patente.

## **III. HECHOS**

### **3.1. REVOCATORIA DIRECTA POR INDEBIDA NOTIFICACIÓN**

3.1.1. El día 27 de Julio de 2011 radiqué ante la SIC, poder a mí conferido para continuar como apoderada en el expediente de la referencia.

3.1.2. El día 31 de Agosto de 2011 mediante Resolución No. 47353 se denegó el privilegio de patente a la invención "DISPOSICION PARA SEPARAR ESPERMATOZOIDES CONGELADOS EN POBLACIONES QUE CONTIENEN CROMOSOMAS X Y CROMOSOMAS Y" ordenando notificar de esta decisión a la Dra Luz Clemencia de Páez.

3.1.3. Frente a la antedicha decisión, el día 30 de Septiembre de 2011 y el 05 de Octubre de 2011 radiqué escrito solicitando que se notificara en debida forma la Resolución 47353.

3.1.4. Atendiendo a la anterior solicitud, procedió el Señor Superintendente a emitir la Resolución No. 79407 de Diciembre 30 de 2011 que revoca parcialmente el artículo segundo de la resolución 47353 de Agosto 31 de 2011, en el sentido de ordenar notificarme personalmente el contenido de dicha resolución.

3.1.5. En aplicación de lo ordenado en la Resolución 79407 de Diciembre 30 de 2011, procedí el día 01 de febrero de 2012 a notificarme personalmente de la Resolución 47353 de Agosto 31 de 2011.

3.1.6. Frente al hecho anterior, concurro ante su Despacho para presentar recurso de reposición contra la Resolución 47353 del 31 de Agosto de 2011.

## **3.2. LA DECISIÓN RECURRIDA**

3.2.1. Mediante la Resolución 47353 del 31 de Agosto de 2011, objeto de la presente impugnación, el Superintendente de Industria y Comercio decidió negar el privilegio de patente para la invención "DISPOSICION PARA SEPARAR ESPERMATOZOIDES CONGELADOS EN POBLACIONES QUE CONTIENEN CROMOSOMAS X Y CROMOSOMAS Y".

3.2.2. En la parte motiva de la providencia, se mencionaron los siguientes fundamentos:

### **3.2.2.1. Modificaciones no aceptadas.**

El Superintendente aclara en la Resolución 47353 que las reivindicaciones que fueron estudiadas para emitir la decisión final son las nueve (9) reivindicaciones que se encuentran en los folios 142 a 144 y que fueron radicadas el día 21 de Septiembre de 2010 junto con la respuesta a la oficio 6423 notificado el 14 de mayo de 2010.

El pliego reivindicatorio que contenía veinte (20) reivindicaciones y que fue radicado el día 4 de febrero de 2011 en respuesta al Oficio 14756 de 2010, no fue aceptado por cuanto *"mencionan un método para teñir células de espermatozoides, con diferentes características técnicas, que no había sido revelado inicialmente, razón por la cual las reivindicaciones amplían el objeto de la solicitud"*<sup>1</sup>.

El capítulo reivindicatorio sufrió una nueva modificación según memorial radicado el día 04 de Febrero de 2011 por la solicitante en donde según concepto del Superintendente *"[...] la presentación del mismo capítulo de la solicitud originalmente radicada con el número 03-54828 (solicitud inicial de la cual se derivó la presente divisional). El objeto de este último capítulo*

<sup>1</sup> Resolución 47353 del 31 de Agosto de 2011 página 3 de 10.

consiste en un "método para teñir células de esperma", el cual, aparte de ser materia ya analizada y negada, incluye reivindicaciones que en sus características técnicas no eran parte de la presente divisional, tal y como fue aceptada inicialmente."

### 3.2.2.2. Determinación del estado de la técnica.

Teniendo en cuenta la fecha de prioridad válidamente reclamada del 29 de Noviembre de 2000, ese Despacho procedió a efectuar la búsqueda de documentos del estado de la técnica encontrando la patente estadounidense No. 6.149.867 titulada "*Sheath fluids and collection systems for sex-specific cytometer sorting of sperm*" publicada el día 21 de Noviembre de 2000.

Esta anterioridad US6.149867 (que de ahora en adelante se mencionará como D1) divulga un sistema mejorado de citómetro de flujo, adaptado para ser usado en la selección de esperma por diferenciación del sexo y también describe un proceso de teñido del semen para ser detectado por un sistema de láser que debido a la diferencia en las magnitudes de onda de los cromosomas Y y X, permiten la separación de cada muestra de semen.

### 3.2.2.3. Nivel Inventivo.

La Resolución 47353 del 31 de Agosto de 2011 fundamenta su decisión de negar la solicitud de patente bajo análisis en una supuesta falta de nivel inventivo frente a lo divulgado en D1.

El Superintendente en su resolución emplea los siguientes argumentos como soporte de su decisión final:

*"[...] el método para diferenciar espermatozoides, reivindicado en la presente solicitud carece de nivel inventivo, porque a los ojos de una persona del oficio normalmente versada en la materia técnica se deriva, de manera evidente, a partir de la información enseñada por el estado del arte."*

*"[...] Las diferencias de la solicitud frente a D1, radican en la mayor concentración de colorante (40 – 20500 micro-molar). Estas diferencias, relacionadas con las condiciones a las cuales se realiza el proceso de teñido y de mantenimiento de las células no demuestran un salto sorprendente en la separación de cromosomas, ya que en D1 se logró una eficiencia en la separación del 90% (Col 15, líneas 8 a 10), mientras que en la solicitud la eficiencia para separar poblaciones de cromosomas es de más del 85%".*

*"Por otra parte, puesto que D1 divulgaba previamente la necesidad de separar espermatozoides que contienen cromosomas X de espermatozoides que contienen cromosomas Y e indicaban que cada población resultante tuviera un número significativo de espermatozoides separados de forma correcta e [sic] uniforme, una persona del oficio normalmente versada en la materia, motivada por tal sugerencia, lograría métodos presentados en la solicitud, basándose en la enseñanzas divulgadas en D1".*

Sobre los argumentos presentados por el solicitante en las respuestas a los exámenes de patentabilidad, la Decisión contenida en la Resolución 47353 resalta:

*"[...] tal como se ha determinado en el análisis de patentabilidad, las diferencias de la solicitud y la anterioridad radican en la separación de esperma, previamente congelado y descongelado, o de semen fresco."*

*"Debido a que no se evidencia un efecto técnico sorprendente, y el estado de la técnica indica el separar, mediante una metodología similar a la reivindicada, esperma de variada procedencia, la solicitud continúa careciendo de nivel inventivo[...]"*

*"La característica técnica de congelar y descongelar el semen antes del tratamiento, no evidencia efectos técnicos sorprendentes ligados a diferencias estructurales con fines de separación del esperma, como se demostró en el análisis de patentabilidad. [...]"*

*"El solicitante menciona que "debido a que D1 no revela el proceso de separación de esperma teñido que había sido previamente congelado y posteriormente descongelado, una persona versada en la materia haría caso omiso en cuanto a que el esperma congelado y posteriormente descongelado pudiera ser separado" (Fl. 175) "*

*"El anterior argumento es cierto, en la medida en que la solicitud prueba efectivamente que es posible separar semen previamente congelado y descongelado, pero el procedimiento para realizarlo es el mismo que el divulgado por el documento del estado de la técnica (D1). Según este argumento, dicha característica resultaría ser un objeto a lograr del método y no un paso necesario para la efectiva separación del material cromosomal, cuya separación da el mismo resultado y no una ventaja técnica para el producto final."*

*"(...) los valores empleados de tinción de concentración de colorante, tiempo y temperatura son muy similares, siendo, de hecho, menores en la anterioridad, demostrándose buena cantidad (90%) en la viabilidad. Por tanto, el problema que se plantea en este argumento ya estaba resuelto en D1, tal como se observó en el análisis de patentabilidad."*

*"En consecuencia, el método reivindicado carece de nivel inventivo, ya que se deriva de manera evidente del estado del arte y resulta obvio para una persona normalmente versada en la materia técnica [...]"*

#### **IV. FUNDAMENTOS DEL RECURSO**

Respetuosamente me permito disentir de lo expuesto por el Despacho en la Resolución 47353, objeto del presente recurso, mediante la cual se deniega el privilegio de patente a la invención de la referencia por falta de nivel inventivo.

Lo anterior por las siguientes razones jurídicas y técnicas sobre las cuales respetuosamente solicitamos un pronunciamiento expreso de su Despacho:

##### **4.1. LA RESOLUCIÓN 47353 VIOLA POR INTERPRETACIÓN ERRÓNEA LOS ARTÍCULOS 14 Y 18 DE LA DECISIÓN 486 DE LA COMISIÓN DE LA COMUNIDAD ANDINA**

De acuerdo con los artículos 14 y 18 de la Decisión 486:

*"Artículo 14.- Los Países Miembros otorgarán patentes para las invenciones, sean de producto o de procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial."(Subrayas fuera del texto original).*

*"Artículo 18.- Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa*

invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica" (Subrayas fuera del texto original)

Antes de explicar las razones por las cuales la presente invención si cumple con el requisito de nivel inventivo, encuentro conveniente reflexionar sobre el criterio de nivel inventivo.

#### 4.1.1. El requisito de nivel inventivo

El artículo 18 de la Decisión 486 establece que una invención demuestra nivel inventivo si no resulta obvia o no se deriva de manera evidente del estado de la técnica para una persona medianamente versada en el arte.

Para que una invención resulte sin nivel inventivo, su objeto deberá ser obvio o derivable de manera evidente, con lo cual dicho objeto de la invención no necesitaría de prueba alguna para saber su resultado, o bien porque existen instrucciones específicas para su obtención, o porque existen sugerencias claras y precisas que lleven a cualquier persona a la misma posibilidad de solución.

De esta manera, cuando el resultado objeto de la invención sea el efecto de una investigación en donde no hubo instrucciones específicas que apunten al resultado que se evalúa, o cuando existiendo sugerencias sobre dicho posible resultado, existía la posibilidad de que dichas sugerencias pudieran producir otros resultados, se comprobará el cumplimiento del requisito del nivel inventivo.

En cuanto a la persona medianamente versada en la técnica, se entiende como aquel profesional que tenga alguna experiencia en ese campo técnico, sin que sea un experto en dicha materia con posgrados o muchos años de experiencia.

Adicionalmente, en relación con el alcance del requisito de nivel inventivo, el Tribunal de Justicia de la Comunidad Andina ha establecido lo siguiente:

*"Como segundo requisito, adicional al de la novedad, se exige que la invención tenga nivel inventivo, esto es, que implique un salto cualitativo en relación con la técnica existente. Este requerimiento, previsto por el artículo 4 de la Decisión 344, presupone que la invención, además de no ser obvia para una persona del oficio normalmente versada en la materia técnica, debe ser siempre el resultado de una actividad creativa del hombre, lo que no impide que se alcance la regla técnica propuesta utilizando procedimientos o métodos comunes o ya conocidos en el área técnica correspondiente, aunque tampoco debe constituir el resultado de derivaciones evidentes o elementales de lo ya existente para un experto medio en esa materia técnica. "El experto medio deberá poseer experiencia y contar con un saber general en la materia y con una serie de conocimientos en el campo específico de la invención" (Proceso 43-IP-2002, publicado en la G.O.A.C. N° 870 de 9 de diciembre de 2002, caso: FORMULACIONES DE PEPTIDOS HIDROSOLUBLES DE LIBERACIÓN RETARDADA).*  
*(...) En consecuencia, a efectos de examinar el nivel inventivo, la Oficina Nacional Competente se fijará en el estado de la técnica existente y en lo que ello representa para una persona del oficio normalmente versada en la materia; esto es que, a la luz de los identificados conocimientos existentes en el área*

técnica correspondiente, se verá si para un experto medio en esa materia técnica —sin que llegue a ser una persona altamente especializada— pueda derivarse de manera obvia la regla técnica propuesta.<sup>2</sup> (Subrayado fuera de texto).

Es decir, el hecho que un procedimiento o método común ya conocido sea utilizado como mecanismo o punto de partida para llegar a una solución particular, no necesariamente convierte su utilización y los resultados específicos obtenidos en evidentes para una persona medianamente versada en el arte.

Para determinar la existencia de nivel inventivo se tienen que estudiar las circunstancias especiales de cada caso, porque como bien lo señala el Tribunal se pueden lograr resultados inesperados aplicando procedimientos conocidos; es decir, lo que debe ser inesperado es el resultado inventivo que se somete a evaluación para obtener el derecho de patente.

Habiendo aclarado algunos aspectos sobre el concepto del nivel inventivo, se entrará a demostrar cómo no era obvia ni evidente la utilización de procedimientos conocidos en la invención ahora propuesta relacionada con clasificación del semen por cuanto la materia prima, es decir el semen que se iba a someter al proceso, presentaba unas características que hacían suponer que no se obtendrían buenos resultados con la aplicación de los procedimientos conocidos.

#### **4.1.2. El objeto y problemas a solucionar por la invención de XY, LLC.**

Se han dado muchos avances en el campo de mejoramiento genético comenzando desde la inseminación artificial hasta por ejemplo, el trasplante de embriones. Hoy es posible obtener semen con células escogidas para obtener una cría de un género específico que se conoce como semen sexado. Esta posibilidad supone un gran avance debido a que permite, por ejemplo, en una ganadería con propósito lechero, obtener hembras de reemplazo con certeza, lo que da continuidad al programa de mejoramiento genético del ganadero y le permite hacer inversiones más seguras en semen de calidad, sin tener que llevarse la desilusión de obtener un macho después de haber invertido dinero en una inseminación artificial.

Esta tecnología también puede tener amplia aplicación en otras especies como por ejemplo en mamíferos en vía de extinción.

En el texto de la solicitud de patente en el acápite de antecedentes de la invención, la solicitante XY explica que el esperma puede ser recolectado a partir de una gran variedad de mamíferos y luego se puede separar en grupos que contengan cromosomas X (que determina el sexo masculino en humanos) y grupos que contengan cromosomas Y, basados en las diferencias de contenido de ADN. Los cromosomas X tienen más ADN que los cromosomas Y.

<sup>2</sup> Proceso 174-IP-2005. Interpretación prejudicial de los artículos 1, 2 y 4 de la Decisión 344 de la Comisión del Acuerdo de Cartagena formulada por el Consejo de Estado de la República de Colombia, Sala de lo Contencioso Administrativo, Sección Primera. Patente: Procedimiento para reprimir una población de insectos. Actor: RHONE POULENC JARDIN. Proceso Interno N° 2001-0062 (6865).

Los métodos de separación consisten en teñir el contenido de ADN en el esperma con un fluorocromo o tinte que, luego al exponerlo a luz láser fluorescerán, los espermatozoides X, al tener más ADN, se teñirán más con el fluorocromo y por lo tanto, resplandecerán más permitiendo su separación de los espermatozoides Y que brillarán menos. Este método se conoce como citometría de flujo.

Es así como lograr obtener semen sexado que contenga cromosomas X, permitirá que cuando fecunde el óvulo se obtenga una cría hembra (dos cromosomas XX).

A pesar de las manifiestas ventajas de la utilización de este tipo de semen sexado, su utilización no se ha podido difundir por los siguientes inconvenientes:

- Contaminación cruzada en el semen clasificado; Esto quiere decir que se obtiene semen clasificado con cromosomas del otro tipo. Este problema se atribuye a una deficiencia en la uniformidad en la cantidad de fluorocromo unido al ADN espermatozoario. Entonces, un rango en la cantidad de fluorocromo se une al esperma que contiene los cromosomas X y un rango en la cantidad de fluorocromo se une al esperma que contiene los cromosomas Y, cuando estos rangos se solapan, entonces es imposible clasificar el esperma y se produce la contaminación cruzada en las muestras de semen clasificado. Este problema de clasificación por baja uniformidad en la impregnación con el fluorocromo, se ve acentuado cuando la clasificación del semen no se ha hecho inmediatamente después de la recolección, es decir con semen fresco.
- Efecto negativo en la fertilización con semen sexado: La cantidad de fluorocromo unido al ADN y el tiempo que transcurre durante el teñido pueden afectar la viabilidad del esperma resultando en bajas ratas de fertilización.
- Efecto negativo en la rata de escisión de los oocitos<sup>3</sup>: Se ha observado que la rata de escisión de los oocitos fertilizados con el semen teñido, disminuye en un 20% cuando el tiempo de teñido requiere 190 minutos, en comparación a cuando se invierten solamente 60 minutos en esta operación de teñido. Es decir, a mayor tiempo de teñido del semen, menor será la rata de escisión de los oocitos que han sido fertilizados con el semen clasificado.
- El tiempo es factor fundamental en el manejo de semen: Se ha demostrado que el semen criopreservado presenta una mayor capacitación<sup>4</sup>, pero por la criopreservación se acorta su tiempo de viabilidad. De esta manera, las rutinas de teñido del semen deben abreviarse para mantener un número adecuado de células viables en la muestra de semen después de sometida al proceso de clasificación.

<sup>3</sup> Oocito: Célula huevo en desarrollo en cualquiera de dos fases. El oocito primario se deriva de un oogonio por diferenciación cerca del nacimiento; da lugar al oocito secundario poco después de la ovulación, mediante una división del primer cuerpo polar. Se fecunda, el oocito secundario se divide en un ótido y en el segundo cuerpo polar. Tomado del Diccionario enciclopédico de ciencia y tecnología, Tomo III, Editorial Prentice Hall Hispanoamericana, S.A., 1996.

<sup>4</sup> Procesos que permiten que los espermatozoides fertilicen los óvulos en presencia de los fluidos del tracto genital femenino. Tomado del Diccionario enciclopédico de ciencia y tecnología, Tomo I, Editorial Prentice Hall Hispanoamericana, S.A., 1996.

Todos estos inconvenientes para la obtención de semen clasificado han sido superados por el método que se propone en la invención de XY, objeto de la solicitud de la referencia.

Entonces el objeto de la presente invención corresponde con un método para obtener semen sexado viable a partir de muestras de semen que han tenido que ser congeladas por el proceso de criopreservación para su transporte, luego, a partir de este semen congelado se procede a descongelarlo para continuar con los pasos de clasificación; se desea obtener un semen con alta pureza del tipo de cromosoma clasificado, es decir con la más mínima contaminación cruzada con cromosomas del otro tipo y que a pesar de haber sido sometido a este procedimiento, continúe siendo viable y apto para fertilización.

**4.1.3. La anterioridad US 6.149.867 (D1) no afecta el nivel inventivo de la presente invención por cuanto no era deducible o evidente que el método para clasificación de semen que se presenta en D1 sirviera utilizando semen descongelado, que había sido previamente criopreservado, como inicio del proceso de clasificación.**

La anterioridad US 6.149.867 D1, en resumen revela un proceso que corresponde a una forma de separar cromosomas X de cromosomas Y de una muestra de semen, mediante un citómetro de flujo.

Ha reconocido su Despacho que una de las diferencias principales entre la solicitud de patente bajo análisis y la patente D1 es que en esta última hacen la clasificación usando semen fresco, mientras que en la invención de XY se hace a partir de un semen que ha sido previamente congelado y luego descongelado.

Sin embargo, contrario a lo que se afirma en la Resolución 47353, esta diferencia si conlleva un efecto técnico sorprendente como se explicará a continuación:

**4.1.3.1. El alcance de lo publicado en la patente US 6.149.867 (D1).**

La anterioridad US 6.149.867 (D1) muestra un sistema para clasificar células de esperma para determinar su sexo a través de la citometría de flujo. En D1 el fluido envolvente que se utiliza en la citometría de flujo es reemplazado por un fluido que minimiza el estrés de las células de esperma cuando se clasifican y también se mejora el sistema de recolección, todo con el propósito de disminuir el impacto del procedimiento en la viabilidad de las células del semen. En D1 se hace mucho énfasis en que las células de esperma están clasificadas en un tipo de células que no son reparables, y que deben manipularse delicadamente porque mueren muy fácilmente; de esta manera, deben ser tratadas de manera especial para conseguir que después del proceso de clasificación aún queden algunas células viables para su posterior uso en la fertilización.

La primera reivindicación del documento D1 se refiere a un sistema de citometría de flujo para aislar células deseadas que comprende siete componentes, todos ellos enfocados al sistema de citometría sin referirse a las características de recolección de la muestra, ni mencionar las condiciones en las cuales la muestra llega al sistema.

#### **4.1.3.2. El procedimiento de criopreservación (congelación) y los resultados obtenidos en D1.**

Para la preservación de las células de espermatozoides o semen, se hace necesario someterlas a temperaturas muy bajas para conservar esta muestra el mayor tiempo posible sin que pierda su calidad, lo cual se conoce como criopreservación (congelación). Este procedimiento utiliza nitrógeno líquido, en donde la muestra se sumerge en tanques con este nitrógeno líquido, alcanzando temperaturas de hasta  $-195,8^{\circ}\text{C}$  (la temperatura de ebullición del nitrógeno líquido) para que las muestras puedan ser recolectadas y luego distribuidas alrededor del mundo.

En Colombia, se pueden adquirir muestras de semen proveniente de Canadá por ejemplo, con la única condición que se conserven con la criopreservación durante todo su transporte, desde el embarque en Canadá hasta la llegada a la finca del usuario final, quien también deberá tener un tanque con nitrógeno para almacenar las pajillas de semen que ha adquirido. Solo minutos antes de la inseminación se saca el semen del tanque de nitrógeno se lleva a temperatura de aproximadamente  $35^{\circ}\text{C}$  y se insemina la vaca.

En la obtención del semen sexado, se ha demostrado que el mismo proceso de clasificación por medio de citometría de flujo somete la muestra a estreses que hacen que la muestra pierda mucha efectividad en la fecundación del óvulo, y es por esto que se desarrollaron métodos como el de la anterioridad D1, para disminuir los efectos negativos de la clasificación en el producto final y así hacer la inseminación con semen sexado cada vez más efectiva.

La misma congelación (criopreservación) y posterior descongelación del semen para su aplicación en una hembra es de por sí un procedimiento que afecta las células del espermatozoides, entonces cuando se buscaba obtener semen sexado lo importante era evitar que el semen sufriera por criopreservación, descongelación para su clasificación, posterior criopreservación para venta y distribución y nuevamente descongelación para la aplicación en una hembra.

Es así como el procedimiento divulgado en D1 utiliza semen fresco, entendiéndose por semen fresco aquel que ha sido recolectado y clasificado casi inmediatamente, sin someterlo al proceso de criopreservación, con el único propósito de disminuir los estreses en la muestra para así conseguir un producto final con más células viables para la aplicación.

En el cuadro de la página 6 de la Resolución 47353 se indica que lo que se menciona para efectos de transporte en los ejemplos 1 a 3, es equivalente a obtener células de espermatozoides mediante descongelar semen previamente congelado. A continuación, ese Despacho asevera:

*"Los experimentos llevados a cabo en D1 involucran el congelamiento y descongelamiento del semen con fines de su transporte. Dicho mecanismo, por lo tanto, es común en el estado de la técnica, antes o después de la tinción."*<sup>5</sup>

Se presenta un error en el análisis de esta información por cuanto los ejemplos 1 a 3 no muestran que el semen objeto del procedimiento especificado en D1 sea congelado y luego descongelado. Precisamente lo que muestran en dichos ejemplos son ensayos hechos en el semen sexado obtenido con el procedimiento de D1 y lo comparan con semen sin clasificar que ha sido congelado y con una muestra de semen liquido sin congelar.

Es decir, la única referencia que hacen a semen congelado en dichos ejemplos es para efectos comparativos y precisan que el semen sexado según el metodo de D1 solo ha sido "refrescado o enfriado" a 5°C pero sigue siendo semen fresco, no criopreservado como el de la presente invención.

La columna 15 línea 2 del documento D1 dice que se recolectó "semen fresco" de tres toros de 14 a 26 meses de edad. En este ejemplo 1 se describe el procedimiento de clasificación de este semen fresco, el cual, luego de clasificado, se empaca en pajillas que fueron transportadas a una temperatura entre 3 y 5°C en una nevera por 240 km para inseminación entre 5 y 9 horas después de la clasificación (ver columna 15 líneas 20 a 23). A esta temperatura de 3 y 5°C lo que se hace es refrescar (en inglés *cool*) el semen sexado. Luego, en la línea 5 de la misma columna, señalan que como muestra control estándar se toma semen de los mismos toros que ha sido congelado (*frozen* en inglés).

La tabla que se muestra en la columna 15 entre líneas 37 a 44 hace una comparación de resultados con: a) el semen fresco clasificado según el procedimiento D1, b) un líquido de control o referencia y c) semen congelado (*frozen*) sin clasificar.

Precisamente estos resultados evidencian aún más las dificultades de obtener semen sexado viable a partir de semen congelado. Presento a continuación dicha tabla del documento D1 en español:

| <i>Tratamiento</i>       | <i>No. Novillas<br/>inseminadas</i> | <i>No. Novillas<br/>preñadas al día 31-34</i> | <i>No. Novillas preñadas<br/>al día 64-67</i> | <i>Número de fetos<br/>hembra</i> |
|--------------------------|-------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------|
| <b>Semen sexado</b>      | 45                                  | 20 (44%)                                      | 19 (42%)                                      | 18 (95%)                          |
| <b>Control líquido</b>   | 28                                  | 15 (54%)                                      | 15 (54%)                                      | 8 (53%)                           |
| <b>Control congelado</b> | 29                                  | 16 (55%)                                      | 15 (52%)                                      | 12 (80%)                          |

Entonces, a partir de los datos de la tabla contenida en D1 se tiene que a los 64 – 67 días de hecha la inseminación artificial el semen no sexado congelado obtuvo un 52% de preñez y el liquido seminal de control obtuvo un 54% de preñez, frente al 42% del semen sexado sin

<sup>5</sup> Resolución 47353 del 31 de Agosto de 2011, segundo párrafo de la página 7 de 10.

congelación, es decir es más efectivo en cuanto a preñez el semen sin clasificar y que no ha sido sometido a ningún proceso de congelación.

Este ensayo comprueba que las manipulaciones del semen, sea por congelación o por el proceso de clasificación, disminuyen la eficacia del semen en la preñez de hembras inseminadas con el semen alterado (bien sea por congelación o por clasificación).

Por consiguiente, a partir de la lectura de esta prueba una persona versada en la materia técnica correspondiente podría concluir que entonces sumar ambas alteraciones al semen, es decir a un semen congelado pretender clasificarlo, bajaría su eficacia significativamente, lo cual no lo haría viable para su utilización en fertilización artificial.

No obstante lo anterior, se debe precisar que el semen sexado, aunque con la menor rata de preñez, produjo que el porcentaje de hembras fuera de 95%.

El ejemplo de D1 simplemente muestra resultados comparativos con semen no sexado, ni congelado (ver columna 15 líneas 57 a 58). Evidentemente, la rata de preñez con semen fresco, sin ninguna alteración por congelación o por clasificación, es más alta que con semen alterado.

El ejemplo 3 de la misma patente D1 es revelador en cuanto a la incidencia de la temperatura en el manejo de semen. Explica el experimento del ejemplo 3, que se recolectó semen y fue transportado 160 km (aproximadamente 2 horas) hasta el laboratorio de clasificación de cromosomas. En dicho ejemplo se explica que la clasificación se hizo a temperatura ambiente (ver columna 16 línea 30), y luego las pajillas con el semen clasificado fueron enviadas por avión a Colorado, unas a temperatura de 5°C (el texto dice *cooled during shipping* es decir, apenas refrescadas o enfriadas a 5°C durante el transporte, no congeladas) y otras a temperatura ambiente. Explica dicha patente que ninguna de las vacas inseminadas con el semen sexado a temperatura ambiente quedó preñada, pero el 41% de las inseminadas con el semen sexado y enfriado quedaron preñadas.

A partir de todos estos experimentos, una persona versada en la materia podría concluir que usar semen sexado fresco, pero mantenido a una temperatura de 5°C, otorga la mayor rata de preñez (efectividad); porque si se transporta a temperatura ambiente, se pierde completamente la viabilidad del semen sexado y si se congela, sufre otra alteración que se supone baja mucho su efectividad.

Otras conclusiones que se pueden obtener del documento D1 son:

- Si es posible hacer inseminaciones con semen sexado, pero las ratas de preñez varían mucho o se ven afectadas cuando el semen se debe transportar por largas distancias (ver columna 17 líneas 5 a 7).

- En la columna 15, líneas 2 a 8 del documento D1, se muestra que el esperma separado en un 90% es un esperma fresco que se incubó en un medio Piruvato Lactato Albúmina Tirode (TALP por su sigla en inglés) ver específicamente la columna 13, línea 13 de D1. Debe tenerse en cuenta que los buffers basados en TALP son generalmente transparentes, aunque se puede complementar con colorante rojo para suprimir la fluorescencia de las células muertas. El documento D1 sólo se refiere al semen congelado como una muestra control, para el análisis de resultados, y sólo describe el semen congelado "*de acuerdo con estándares de la industria*". En agudo contraste con el TALP transparente que se adiciona al esperma fresco antes de su clasificación, una muestra de esperma congelado "*de acuerdo con estándares de la industria*" generalmente incluirá 20% de yema de huevo, además de un crioprotector como el glicerol (véase la tabla 2 del artículo "Storage of bovine semen in liquid and frozen state" R. Vishwanath y P. Shannon Anexo 2). Estas sustancias no están presentes en el esperma fresco o esperma fresco en un medio TALP. Como punto inicial, por lo tanto, se debe reconocer que el esperma fresco y esperma congelado-descongelado no son idénticos.

Finalmente, a partir de los resultados que se muestran en el ejemplo 3 de D1, se puede llegar a preguntar:

¿Por qué los investigadores de la patente de D1, expertos en la obtención de semen sexado, no transportaron el semen sexado a temperaturas de criopreservación (congelado) y de esta manera, no perder todo su trabajo de clasificación y envío por avión de muestras (en una carrera contra el tiempo), para que después lamentablemente no quedara ninguna hembra preñada?

La respuesta es simple. Para la época del desarrollo del proceso de la patente D1 era muy importante evitar someter el semen a cualquier cambio brusco de temperatura y de condiciones para que se lograra conseguir un semen clasificado (sexado) que fuera eficaz en la fecundación, era evidente que: a) tomar las muestras de semen congeladas b) descongelarlas, c) clasificarlas por el procedimiento de citometría de flujo, d) volverlas a criopreservar, e) transportar y f) volver a descongelar para su aplicación, supondría una combinación de condiciones que no soportaría las frágiles células del semen, y por esta razón, se hicieron todos los esfuerzos que se ilustran en D1 para hacer la clasificación e inseminación tan pronto como fuera posible utilizando semen fresco.

#### **4.1.3.3. Declaración del Dr. Rick Lenz sobre la invención propuesta por XY y la invención contenida en la patente D1.**

Se anexa la declaración hecha por el Dr. Rick Lenz que tiene una experiencia de 28 años en el manejo de semen, es especialista en el campo de la biología reproductiva animal y tiene una licenciatura en Ciencia Animal de la Universidad de Wisconsin-Platteville; quien respondió

algunas preguntas relacionadas con la invención propuesta por XY y la invención contenida en la patente D1. (Anexo 4)

Se le solicitó al Dr. Lenz que indicara algunos de los parámetros típicos de un semen recién recolectado y explicó que en el caso de bovinos una eyaculación sin tratar puede ser:

- 5 a 6 ml de  $>1 \times 10^9$  espermatozoides/ml.
- Motilidad<sup>6</sup> de 75-80% con 20-25% muertos.

En el documento D1 se muestra como el semen recolectado tenía  $1.5 \times 10^9$  espermatozoides/ml (columna 13 líneas 5 y 6) y una motilidad de 60 y 70% (columna 13 líneas 10 a 12).

En su respuesta sobre los parámetros típicos de una muestra de semen congelada-descongelada explicó:

El espermatozoide congelado se almacena en bombillas (también conocidas como pajillas) a  $20 \times 10^6$  espermatozoides/bombilla hasta  $40 \times 10^6$  espermatozoides/bombilla en una bombilla de 0.5 ml. En cuanto a motilidad se refiere, el espermatozoide congelado tendrá menos de 65% de motilidad y un 35% de células muertas.

Entonces, es claro que en D1 se está utilizando semen fresco sin tratar con criopreservación.

Tal y como se explicó anteriormente, otra evidencia que hace precisar que el semen utilizado en D1 es fresco, está relacionada con los componentes de la muestra de semen a ser seleccionada. Para permitir su congelación el semen contiene una solución amortiguadora que comprende 20% de yema de huevo y 6% de glicerol (ver respuesta pregunta 4 de la declaración del Dr. Lenz); de la lectura del documento D1 se evidencia que la composición de la muestra a ser seleccionada no contiene yema de huevo ni glicerol. Específicamente, en el documento D1 se explica que en aplicaciones de la invención para bovinos se puede adicionar 20% de yema de huevo en una solución de citrato **inmediatamente después del teñido** (ver columna 13 líneas 24 a 26).

El Dr. Lenz en su declaración explica las dificultades que se tienen al intentar teñir muestras de semen que han sido previamente congeladas y que por lo tanto, contienen 20% de yema de huevo y 6% de glicerol; debido a que, la presencia de yema de huevo en la muestra hace que tenga un efecto negativo en el teñido de la muestra<sup>7</sup> por cuanto reduce la eficiencia con la cual el colorante se asocia con el ADN nuclear del espermatozoide, lo cual incide en la uniformidad de la tinción y puede aumentar el periodo de incubación y el glicerol también afecta negativamente la viabilidad de la muestra.

<sup>6</sup> La **motilidad** es un término de la biología para expresar la habilidad de moverse espontánea e independientemente.

<sup>7</sup> Ver respuesta a la pregunta 6 de la declaración en el Anexo 4

Por estas razones, los investigadores en D1 realizaron la separación cromosómica en semen fresco y no con semen que había sido previamente congelado y después descongelado para hacer la citometría de flujo.

**4.1.3.4. El procedimiento de D1 es más eficiente en cuanto a la separación de células que el procedimiento de la presente invención por cuanto es más dañino para el semen clasificarlo cuando ha sido previamente sometido a congelación y luego descongelación para su clasificación. Sin embargo clasificar semen congelado-descongelado resulta un salto sorprendente en el estado de la técnica.**

Afirma su Despacho que la eficiencia en la separación en D1 es del 90% (según se muestra en la columna 15 líneas 8 a 10), mientras que en la de la presente solicitud es de más del 85%. Sobre este aspecto considero que no hay ninguna base para realizar esta comparación. Precisamente, la diferencia de porcentaje de eficiencia en la separación en un método y otro, hace manifiesto el hecho de que las diferencias entre hacer una clasificación con el semen fresco es más fácil que hacerla con semen congelado-descongelado, lo cual no ha sido reconocido por ese Despacho.

Precisamente, esta diferencia en el porcentaje de eficiencia en la separación se debe a que el nivel de estrés que sufren las células en D1 es sustancialmente inferior al que sufren las células en el proceso descrito en la presente solicitud. Es obvio que al clasificar células con semen fresco como en D1 la eficacia en la clasificación y viabilidad de las células es superior respecto de la clasificación en semen que primero fue congelado y luego descongelado como en la presente solicitud se describe. Esto es lo que lleva a mi representada a insistir en el cumplimiento de la altura inventiva de la presente invención.

Así mismo, siguiendo lo que se explicó sobre los aditivos en el semen congelado, la adición de yema de huevo puede afectar la capacidad de que los espermatozoides se impregnen de la sustancia colorante de manera uniforme. Sin un alto grado de coloración uniforme, es imposible distinguir la diferencia entre cromosomas X y Y, precisamente se ha comprobado que en bovinos el espermatozoide que porta el cromosoma X tiene 3.8% más ADN que el espermatozoide que porta el cromosoma Y. En segundo lugar, la yema de huevo o la leche descremada (también utilizada en estas soluciones para semen), dado que nos son transparentes, pueden interferir con la capacidad de los detectores para medir con precisión la emisión de fluorescencia de las células espermáticas activadas en un clasificador.

Es bien sabido que tanto el proceso de selección y el proceso de congelación son perjudiciales para los espermatozoides. Por ejemplo, la congelación y descongelación de espermatozoides tiende a matar a cerca de 50% de la población de espermatozoides (Ver en la página 35 de Vishwanath, Anexo 3). Esto se debe en gran parte debido a la expansión de hielo intracelular, a medida de que el agua intracelular se congela, dañando de esta manera los componentes intracelulares suaves adyacentes. Del mismo modo, los esfuerzos mecánicos y químicos

descritos en D1 son perjudiciales para los espermatozoides. Como un ejemplo específico, la muestra que contiene el flujo de espermatozoides debe ser presurizada con el fin de formar gotas de un tamaño apropiado como una frecuencia apropiada. El documento de patente WO2004/012837 (Anexo 4) demuestra que el esperma clasificado a cada presión de 30, 40 y 50 PSI ha aumentado el porcentaje de espermatozoides muertos y ha reducido la motilidad del esperma (ver Tabla 1). Como otro ejemplo de que cualquier operación llevada a cabo en el esperma afecta su porcentaje de espermatozoides muertos, se puede mencionar que la dilución de la muestra durante la inyección de aire en el clasificador de citómetro de flujo produce un impacto negativo en la motilidad de los espermatozoides.

De esta manera, el esperma congelado-descongelado no sería un punto de partida obvio para la clasificación de esperma y, más concretamente que el esperma congelado-descongelado no es el mismo que el esperma fresco.

Igualmente, deseamos reiterar que la persona versada en la materia creía anteriormente que el esperma debilitado por la criopreservación no produciría muestras clasificadas viables en vista del stress químico, físico, choque de pH, choque térmico y choque osmótico que debía soportar durante el proceso de separación. Además, el esperma congelado-descongelado tiene una concentración diferente en comparación con el esperma fresco, lo que hace que la tinción uniforme del esperma congelado-descongelado se haga mucho más difícil.

Por lo tanto, con el fin de hacer la tinción a la parte más densa del esperma congelado-descongelado, una persona versada en la materia se vería inclinada a teñir por tiempo más prolongado o a concentración mas altas de tintura. Sin embargo, el proceso de tinción es también perjudicial para el esperma, por consiguiente, el incremento de tanto el tiempo de tinción como la concentración de la tintura, tienen un impacto negativo en las características de fertilidad del esperma. En vista del stress descrito anteriormente, una persona versada en la materia no habría pensado que una muestra de esperma viable se podría producir a partir de esperma congelado, que fue sujeto a niveles de tinción elevados y que luego se separó de manera exitosa.

Por las razones antes expuestas es que el porcentaje de eficiencia demostrado en el proceso de la solicitud de patente es menor al reportado en D1, pero indiscutiblemente es alto frente a lo que un experto en la materia podría esperar.

#### **4.1.3.4. Los tiempos y concentraciones de colorante utilizados en el proceso de la presente invención difieren mucho de los reportados en D1.**

Deseo reiterar que es un error afirmar que, esencialmente, porque el esperma ha sido congelado antes y posteriormente utilizado para la inseminación, sería obvio separar esperma congelado y luego volverlo a congelar para su distribución. Es decir, no es un punto de partida obvio para este proceso utilizar esperma congelado-descongelado para la clasificación, se debe

tener en cuenta que aparte de quizás algunas aplicaciones diagnósticas, el uso final de los espermatozoides en general, será para fertilización *in vitro* o *in vivo*, lo cual requiere de unas muestras con alta eficacia.

No hay sugerencia en el arte previo que permita dilucidar que se podría descongelar semen previamente congelado y a continuación, clasificarlo y **posteriormente** intentar una fertilización *in vitro* o *in vivo* con este semen clasificado que proviene de un procedimiento que incluyó su descongelamiento. Simplemente porque el paso de la fertilización se lleva a cabo después de otros pasos del proceso, no quiere decir esto que sea posible que aquellos otros pasos del proceso se puedan llevar a cabo antes de la etapa de clasificación. En otras palabras, en este procedimiento, el orden de los factores (en este caso pasos), si afecta el producto.

Además, la técnica de criopreservación de espermatozoides comenzó en la década de 1950. La técnica de clasificación de células activadas por fluorescencia FACS (por su sigla en inglés *Fluorescence Activated Cell Sorting*) y en concreto de clasificación de espermatozoides a través de la citometría de flujo ha comenzado antes de 1991 (ver fecha de presentación de la patente estadounidense US 5.135.759). A pesar de la antigüedad de la técnica de congelación de muestras de espermatozoides y la técnica relativamente antigua de obtener espermatozoides sexados, no había sido demostrado posible la obtención de espermatozoides sexados a partir de espermatozoides previamente congelados solamente hasta la ejecución de la presente invención en el año 2000.

De hecho, en el momento de la invención, se creía que la clasificación de espermatozoides descongelados no iba a funcionar. Cada paso de congelación y clasificación es perjudicial para los espermatozoides. Los expertos en ese momento no creían que el espermatozoides pudiera sobrevivir tanto en el proceso de congelación y luego, el proceso de clasificación lo suficientemente bien como para su uso posterior en la fertilización.

La Tabla 1 de la solicitud de XY muestra la contribución de esta solicitud al estado de la técnica con relación a los tiempos de tinción y las concentraciones de la tintura para alcanzar alta pureza en la separación de células de espermatozoides mientras se mantiene la viabilidad de las mismas. Dicha tabla provee la información necesaria relacionada al desarrollo embrionario, lo que evidencia que las concentraciones de la tintura y el tiempo de tinción allí utilizados dieron células de espermatozoides separadas viables. Se puede concluir que el factor predominante en la tasa de escisión de los oocitos fertilizados con el espermatozoides teñido disminuye en un 20% cuando el tiempo de teñido requiere 190 minutos que cuando se invierte 60 minutos en esta operación, entonces se preferirá utilizar concentraciones de colorante altas por ejemplo 2240  $\mu\text{M}$  con lo cual se invertirá solamente 60 min en el teñido, que emplear las concentraciones cercanas a 40  $\mu\text{M}$ .

Sin embargo, sobre las concentraciones es importante observar que, a pesar que la reivindicación 1 de la invención plantea el rango de 40 – 2500  $\mu\text{M}$ , este rango se limita a 224

$\mu\text{M}$  (reivindicación 3) y a 2240 (reivindicación 4) valores estos que se alejan mucho más del valor de 38  $\mu\text{M}$  reportado en D1 (ver ejemplos), esta diferencia no puede ser considerada como una mera optimización de parámetros.

#### **4.1.3.6. En conclusión, lo publicado en D1 no afecta el nivel inventivo de la presente invención.**

La ventaja técnica obtenida por el hecho de obtener esperma viable, el cual había sido previamente congelado y descongelado, después de ser separado mediante el proceso de la presente solicitud, es clara, por lo que el nivel inventivo de la presente invención debe ser reconocido. Lo anterior, toda vez que cada uno de los pasos de congelamiento de esperma, tinción de esperma y separación de esperma proporcionan varias tensiones y choques sobre las células de esperma relativamente delicadas.

En resumen, el estado de la técnica revela un método para separar cromosomas X de cromosomas Y a partir de semen fresco. Por lo tanto, una persona versada en la materia tendría como expectativa que a partir del estado de la técnica la separación de semen previamente congelado y luego descongelado daría como resultado espermatozoides con una baja viabilidad. Sin embargo, deseamos aclarar que la presente solicitud proporciona, de manera inesperada, que esta separación pueda ser llevada a cabo de manera exitosa, lo que demuestra que la presente invención involucra claramente altura inventiva.

Así entonces, podemos concluir que la Resolución impugnada fue expedida con violación del artículo 18 de la Decisión 486 debido a que no era posible derivar de manera obvia la presente invención a partir del documento más cercano del estado del arte encontrado por su Despacho (US 6.149.867).

#### **4.2. EL NIVEL INVENTIVO DE LA INVENCION FUE RECONOCIDO POR OTRAS OFICINAS DE PATENTE.**

Aunque entiendo que las concesiones de patentes en oficinas de otros países no son vinculantes para la SIC para que tome una decisión en el mismo sentido, no se puede desconocer que, siendo los requisitos de novedad, nivel inventivo y aplicación industrial mundialmente aplicados por todas las Oficinas de Patentes para otorgar o negar solicitudes, se haya otorgado el título de patente para esta misma invención en Estados Unidos con los números US 7,713,687 y US 7,771,921, en Argentina con el número AR 036869B1 y en Australia con los números de patente 2002237689 y 2008200810 (**Anexo 5**).

#### **4.3. MODIFICACIÓN A LAS REIVINDICACIONES POR ERROR EVIDENTE EN LA DEPENDIENCIA DE LAS REIVINDICACIONES.**

Aprovecho la oportunidad para presentar un capítulo reivindicatorio modificado que consta de 9 reivindicaciones y que corresponde exactamente al capítulo reivindicatorio evaluado por ese Despacho para emitir esta decisión, pero que ha sido enmendado en cuanto a la dependencia de las reivindicaciones, la cual contenía errores evidentes que requerían su corrección.

Presento en el Anexo 1 el formulario de modificación por error material, el nuevo capítulo reivindicatorio y la tasa correspondiente a esta modificación.

#### 4.4. ANEXOS

Anexo 1. Nuevo capítulo reivindicatorio modificado, formulario de modificación y pago de la tasa por modificación.

Anexo 2. Declaración Dr. Rick Lenz.

Anexo 3. Artículo "Storage of bovine semen in liquid and frozen state" R. Vishwanath y P. Shannon.

Anexo 4. Publicación de patente WO2004/012837

Anexo 5. Copia de la primera página de la publicación de patentes en paralelas para Estados Unidos US 7,713,687 y US 7,771,921, Argentina AR 036869B1 y Australia 2002237689, 2008200810 A1 y 2008200810B2.

Anexo 6. Copia de la Resolución No. 47353 notificada el 01 de Febrero de 2012.

#### V. NOMBRE Y DIRECCION

El nombre y la dirección de la sociedad recurrente es el siguiente:

**XY, LLC.**

22575 State Highway 6 South,

Navasota,

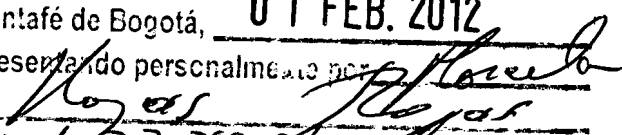
Texas 77868,

Estados Unidos de América

#### VI. NOTIFICACIONES

Atentamente manifiesto a usted que recibiré notificaciones en la Secretaria de su Despacho o en mi oficina de abogada situada en la Carrera 13 No. 29 – 41/21, de esta ciudad de Bogotá, D.C.

SUPERINTENDENCIA DE SEÑOR Superintendente  
Santafé de Bogotá, 01 FEB. 2012

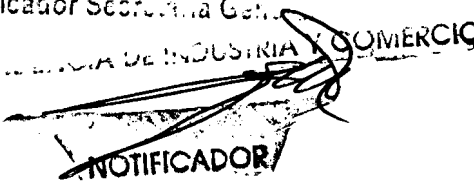
Presentando personalmente por   
C.C. 1832387271  
T.P. 191426

  
**Marcela ROJAS ROJAS**

CC. 1.032.387.271

T.P. 191.426

Notificador Secretaría General

SECRETARIA DE INDUSTRIA Y COMERCIO  
  
**NOTIFICADOR**

**ANEXO 1. NUEVO CAPÍTULO REIVINDICATORIO MODIFICADO, FORMULARIO  
DE MODIFICACIÓN Y PAGO DE LA TASA POR MODIFICACIÓN.**

**REPUBLICA DE COLOMBIA**  
**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO**

**SUPERINTENDENCIA DELEGADA PARA LA**  
**PROPIEDAD INDUSTRIAL**

**DIVISIÓN DE NUEVAS CREACIONES**

**SOLICITUD DE INSCRIPCIÓN DE**  
**MODIFICACIONES A SOLICITUD EN**  
**TRÁMITE**

**NÚMERO DE EXPEDIENTE: 3-54828A**

**Patente: DISPOSICION PARA SEPARAR**  
**ESPERMATOZOIDES CONGELADOS EN POBLACIONES**  
**QUE CONTIENEN CROMOSOMAS X Y CROMOSOMAS Y**

**SOLICITANTE: XY, LLC.**

**DOMICILIADO EN: Navasota, Texas, Estados Unidos**  
**de América**

**APODERADO: Marcela ROJAS ROJAS**

**Bogotá D.C., Enero de 2012**

Superintendencia de  
Industria y Comercio

**FORMULARIO ÚNICO DE CORRECCIONES Y MODIFICACIONES**

**SOLICITUD DE:**

1 ☐ Corrección de Error Material

☒ Modificación a una Solicitud

|                                |                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                                                                             |
|--------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>2</b><br><b>SOLICITANTE</b> | <b>Nombre:</b> XY, LLC.<br><b>Dirección:</b> 22575 State Highway 6 South,<br>Navasota, Texas 77868, Estados Unidos de<br>América<br><b>Nacionalidad o Domicilio:</b> Navasota, Texas,<br>Estados Unidos de América<br><b>Lugar de Constitución:</b> Navasota, Texas, Estados<br>Unidos de América<br><b>Tel:</b> (571)3230233 <b>Fax:</b> (571)3230234<br><b>E-mail:</b> <a href="mailto:info@brlatina.com">info@brlatina.com</a> | <b>IDENTIFICACIÓN</b><br><br><input type="checkbox"/> C.C. <input type="checkbox"/> NIT<br><input type="checkbox"/> C.E. <input checked="" type="checkbox"/> Otro<br>Número |
|                                |                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                                                                                                                             |

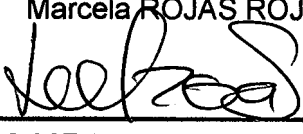
|                                                            |                                                                                                                                                                                                                                                                       |                                                                                                                                                                                                                             |
|------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>3</b><br><b>REPRESENTANTE</b><br><br><b>O APODERADO</b> | <b>Nombre:</b> Marcela ROJAS ROJAS<br><b>Dirección:</b> Carrera 13 No. 29 – 41/21 Of 241,<br>Parque Central Bavaria, Bogotá, Colombia<br><b>Tel:</b> (57 1)3230233 <b>Fax:</b> (571)3230234<br><b>Email:</b> <a href="mailto:info@brlatina.com">info@brlatina.com</a> | <b>IDENTIFICACIÓN</b><br><br><input checked="" type="checkbox"/> C.C. <input type="checkbox"/> NIT<br><br><input type="checkbox"/> C.E. <input type="checkbox"/> Otro<br><b>Número:</b> 1,032,387,271<br><b>T.P:</b> 191426 |
|                                                            |                                                                                                                                                                                                                                                                       |                                                                                                                                                                                                                             |

**4 Número de expediente:** 3-54828A

**5 Descripción de la corrección o modificación solicitada**  
 Solicito se tenga en cuenta para el estudio técnico de la patente de la referencia, el pliego reivindicatorio que adjunto a la presente.

**6 Comprobante de pago No.** 12-0010070 **Fecha** Enero 31 de 2012

**7 Anexos**  
☒ Comprobante de pago de la tasa  
☐ Poderes, si fuere el caso  
☐ Certificado de existencia y representación legal de las partes.

**8 Nombre:** Marcela ROJAS ROJAS  
**Firma:**   
 C.C. 1,032,387,271  
 T.P. 191426

|                                                                                                                                                                                                                                                                                                                              |                                                  |                                                             |              |                                            |            |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------|-------------------------------------------------------------|--------------|--------------------------------------------|------------|
| SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO<br>NIT : 800.176.089-2<br>- / -                                                                                                                                                                                                                                                     |                                                  |                                                             |              |                                            |            |
| <br><b>Industria y Comercio</b><br>SUPERINTENDENCIA                                                                                                                                                                                         | RECIBO DE CAJA                                   |                                                             |              | <b>No. 12 - 0010070</b>                    |            |
|                                                                                                                                                                                                                                                                                                                              |                                                  |                                                             |              | Bogotá D.C., Febrero 01 de 2012 - 14:07:58 |            |
| RECIBIDO DE : B&R LTDA                                                                                                                                                                                                                                                                                                       |                                                  |                                                             |              | NI 830.122.790                             |            |
| *** Soporte del Pago ***                                                                                                                                                                                                                                                                                                     |                                                  |                                                             |              |                                            |            |
| TIPO PAGO                                                                                                                                                                                                                                                                                                                    | BANCO                                            | CUENTA                                                      | No. PAGO     | FECHA PAGO                                 | VR. PAGO   |
| CONSIGNACION                                                                                                                                                                                                                                                                                                                 | BANCO DE BOGOTA                                  | 062754387                                                   | 191646022    | 01/02/2012                                 | 120.000.00 |
| *** Conceptos Pagados ***                                                                                                                                                                                                                                                                                                    |                                                  |                                                             |              |                                            |            |
| CANT.                                                                                                                                                                                                                                                                                                                        | RENTISTICO                                       | CONCEPTO                                                    | Vr.UNDITARIO | Vr.CONCEPTO                                |            |
| 1                                                                                                                                                                                                                                                                                                                            | 50005-01-03 CAMBIO DE MODALIDAD Y MODIFICACIONES | EN N. CREACION CORRECCION A SOLICITUD EN TRAMITE / MODIFICA | 120.000.00   | 120.000.00                                 |            |
|                                                                                                                                                                                                                                                                                                                              |                                                  |                                                             |              | \$120.000.00                               |            |
|                                                                                                                                                                                                                                                                                                                              |                                                  |                                                             | =====        |                                            |            |
| SON: **CIENTO VEINTE MIL PESOS MONEDA CORRIENTE***                                                                                                                                                                                                                                                                           |                                                  |                                                             |              |                                            |            |
| Responsable:                                                                                                                                                                                                                               |                                                  |                                                             |              |                                            |            |
| Recibo de Caja Aplicado al Expediente No. _____                                                                                                                                                                                                                                                                              |                                                  |                                                             |              |                                            |            |
| <div>SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO<br/><br/><b>No. 03-054828-A-00016-0000</b><br/>Fecha: 2012-02-01 14:18:18 Dep. 2020 DIR.NUEVASCR<br/>Tra. 11 PATENTEIYII Eve: 379 FASENACIONALI<br/>Act. 412 REPOSPRESRECU Folios: 80</div> |                                                  |                                                             |              |                                            |            |
| Sede Centro: Carrera 13 No. 27 - 00 Pisos 3,4,5 y 10 Bogotá, D.C.- Colombia<br>Web: www.sic.gov.co e-mail: info@sic.gov.co Conmutador: (571) 5870000 Fax: (571) 5870284 Línea: 018000-910165 Call Center: (571) 6513240<br>Febrero 1 de 2012 - 14:07:59                                                                      |                                                  |                                                             |              |                                            |            |

## REIVINDICACIONES

1. Un método para aislar células de esperma deseadas, que comprende las etapas de:
  - a. obtener células de esperma por medio de descongelar semen previamente congelado;
  - b. incubar las células de esperma con colorante fluorocromo en una concentración micro-molar de entre 40 y 2500 por entre 50 y 200 minutos;
  - c. suministrar dichas células de esperma a un citómetro de flujo;
  - d. crear un entorno de fluido de envainado dentro de dicho citómetro de flujo en el cual dichas células de esperma están retenidas;
  - e. pasar dichas células de esperma a través de una boquilla mientras están retenidas en dicho entorno de fluido de envainado pasando a través de un sistema de detección;
  - f. excitar dichas células de esperma con una fuente de radiación;
  - g. detectar la magnitud de la fluorescencia emitida por el enlace fluorocromo al ADN del esperma con una disposición sensora de células de esperma que responde a dichas células de esperma; y
  - h. separar dichas células de esperma que tienen una característica deseada, basándose en la magnitud de fluorescencia emitida por cada una de las células, con una disposición de separación y discriminación.
2. El método para aislar células de esperma deseadas, tal como se define en la reivindicación 1, 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.
3. El método para aislar células de esperma deseadas, tal como se define en la reivindicación 2, donde dicho mamífero macho comprende dicho bóvido y donde dicha concentración micro-molar de colorante fluorocromo es 224.

4. El método para aislar células de esperma deseadas, tal como se define en la reivindicación 2, donde dicho mamífero macho comprende dicho bóvido y donde dicha concentración micro-molar de colorante fluorocromo es 2240.

5. El método para aislar células de esperma deseadas, tal como se define en la reivindicación 4, donde dicho tiempo de duración es de 190 minutos.

6. El método para aislar células de esperma deseadas, tal como se define en la reivindicación 5, donde dicho tiempo de duración es de 60 minutos.

7. El método para aislar células de esperma deseadas, tal como se define en la reivindicación 1, 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 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%.

8. El método para aislar células de esperma deseadas, tal como se define en la reivindicación 6, que comprende además un elemento de recolección dentro del cual están aisladas las células de esperma que contienen cromosomas X.

9. El método para aislar células de esperma deseadas, tal como se define en la reivindicación 6, que comprende además un elemento de recolección dentro del cual están aisladas las células de esperma que contienen cromosomas Y.

**ANEXO 2. DECLARACIÓN DR. RICK LENZ.**



The State of Texas  
Secretary of State

Requested for use in COLOMBIA

Not for use within the United States of America

This Apostille only certifies the signature, the capacity of the signer and the seal or stamp it bears. It does not certify the content of the document for which it was issued.

**APOSTILLE**

(Convention de La Haye du 5 Octobre 1961)

|                                |                                                                                        |
|--------------------------------|----------------------------------------------------------------------------------------|
| 1. Country                     | United States of America                                                               |
| This public document           |                                                                                        |
| 2. has been signed by          | ADRIENNE C CASTILLO                                                                    |
| 3. acting in the capacity of   | Notary Public, State of Texas                                                          |
| 4. and bears the seal/stamp of | ADRIENNE C CASTILLO,<br>Notary Public, State of Texas,<br>Commission Expires: 06-09-15 |

**CERTIFIED**

|                                       |                       |
|---------------------------------------|-----------------------|
| 5. at Austin, Texas                   | 6. on August 17, 2011 |
| 7. by the Secretary of State of Texas |                       |
| 8. Certificate No. N-812492           |                       |
| 9. Seal                               | 10. Signature:        |



Hope Andrade  
Secretary of State

ST/eg

Declaration of Rick Lenz

1. Describe your technical background in reproductive biology and particularly sex sorting of sperm.

I have been in the semen business for 28 years, and director of laboratory for sorting semen since 2004. I have a Bachelor of Science in Animal Science from the University of Wisconsin-Platteville. I have a Masters degree and a Ph.D. in Reproductive Physiology from the University of Illinois and have held two post doctoral research positions.

2. What composition does the freshly collected semen include in U.S. Patent 6,149,867 to Siedel?

In example 1 of Seidel, 75x10<sup>6</sup> sperm/ml are extended in a TALP buffer with the addition of Hoechst 33342. TALP formulations can vary, but a typical TALP can include:

|                                      |           |
|--------------------------------------|-----------|
| NaCl                                 | 95.0 mM   |
| KCl                                  | 3.0 mM    |
| NaHPO <sub>4</sub>                   | 0.3 mM    |
| NaHCO <sub>3</sub>                   | 10.0 mM   |
| MgCl <sub>2</sub> •6H <sub>2</sub> O | 0.4 mM    |
| Na Pyruvate                          | 2.0 mM    |
| Glucose                              | 5.0 mM    |
| Na lactate                           | 25.0 mM   |
| HEPES <sup>a</sup>                   | 40.0 mM   |
| Bovine serum albumin <sup>b</sup>    | 3.0 mg/ml |
| Gentamycin Sulfate                   | 30 µg/ml  |
| (See Table 3, US Patent 7,820,425)   |           |

3. Describe some typical parameters of freshly collected semen.

Declaración de Rick Lenz

1. Describa su formación técnica en biología reproductiva y en particular en la selección del sexo del esperma

He estado en el negocio del semen durante 28 años y director de laboratorio para selección de semen desde 2004. Tengo una Licenciatura en Ciencias en Ciencia Animal de la Universidad de Wisconsin-Platteville. Tengo un grado de maestría y un doctorado en Fisiología Reproductiva de la Universidad de Illinois y he tenido dos puestos de investigación post-doctoral.

2. ¿Qué composición incluye el semen recientemente recolectado en la patente de E.U.A. 6,149,867 para Siedel?

En el ejemplo 1 de Seidel, 75x10<sup>6</sup> esperm/ml se diluyen en una solución amortiguadora de TALP con la adición de Hoechst 33342. Las formulaciones TALP pueden variar, pero un TALP típico puede incluir:

|                                         |           |
|-----------------------------------------|-----------|
| NaCl                                    | 95.0 mM   |
| KCl                                     | 3.0 mM    |
| NaHPO <sub>4</sub>                      | 0.3 mM    |
| NaHCO <sub>3</sub>                      | 10.0 mM   |
| MgCl <sub>2</sub> •6H <sub>2</sub> O    | 0.4 mM    |
| Piruvato Na                             | 2.0 mM    |
| Glucosa                                 | 5.0 mM    |
| Lactato Na                              | 25.0 mM   |
| HEPES <sup>a</sup>                      | 40.0 mM   |
| Albúmina de suero bovino <sup>b</sup>   | 3.0 mg/ml |
| Sulfato de Gentamicina                  | 30 µg/ml  |
| (Ver Tabla 3, patente de EUA 7,820,425) |           |

3. Describir algunos de los parámetros típicos de semen recientemente recolectado.

In the case of bovine, raw ejaculate can be 5 to 6 ml of  $>1 \times 10^9$  sperm/ml. Sperm motility immediately after collection can be 75-80%, with 20-25% dead (Erickson, 1966). U.S. Patent 6,149,867 described collected sperm at a concentration of  $1.5 \times 10^9$  sperm/ml with a motility of between 60 and 70%.

4. What compositions would typically be included in a frozen thawed sperm sample?

Frozen sperm typically contains a buffer – such as TRIS, 20% egg yolk, and 6% glycerol (US Patent 7,820,425). Both the egg yolk and the glycerol have properties which protect the sperm against cold shock and freezing respectively, while TRIS is a well known buffer as a physiologically acceptable carrier having a pH and osmolarity which promotes bovine sperm longevity.

An exemplary egg yolk TRIS extender can include:

|                              |             |
|------------------------------|-------------|
| Glycerol:                    | 6% vol/vol  |
| Citric Acid:                 | 56 mM       |
| Tris                         |             |
| [hydroxymethyl]aminomethane: | 200 mM      |
| Egg yolk:                    | 20% vol/vol |
| Fructose:                    | 56 mM       |
| Tylosin:                     | 50 µg/ml    |
| Gentamicin:                  | 250 µg/ml   |
| Lincomycin                   | 150 µg/ml   |
| Spectinomycin:               | 300 µg/ml   |

(Table 3 and the following paragraph, US Patent 7,820,425)

En el caso de los bovinos, la eyaculación sin tratar puede ser de 5 a 6 ml de  $>1 \times 10^9$  espermatozoides/ml. La motilidad del espermatozoide inmediatamente después de la recolección puede ser de 75-80%, con 20-25% muertos (Erickson, 1966). La patente de E.U.A. 6,149,867 describió el espermatozoide recolectado a una concentración de  $1.5 \times 10^9$  espermatozoides/ml con una motilidad de entre 60 y 70%.

4. ¿Qué composiciones estarían típicamente incluidas en una muestra congelada de espermatozoide líquido?

El espermatozoide congelado contiene típicamente una solución amortiguadora – tal como TRIS, 20% de yema de huevo, y 6% de glicerol (patente de EUA 7,820,425). Tanto la yema de huevo como el glicerol tienen propiedades que protegen al espermatozoide contra el choque en frío y la congelación respectivamente, mientras que TRIS es una solución amortiguadora bien conocida como un portador fisiológicamente aceptable que tiene un pH y osmolaridad que promueve la longevidad del espermatozoide de bovino.

Un diluyente ejemplar de TRIS para yema de huevo puede incluir:

|                                 |             |
|---------------------------------|-------------|
| Glicerol:                       | 6% vol/vol  |
| Ácido Cítrico:                  | 56 mM       |
| Tris [hidroximetil]aminometano: | 200 mM      |
| Yema de huevo:                  | 20% vol/vol |
| Fructuosa:                      | 56 mM       |
| Tilosina:                       | 50 µg/ml    |
| Gentamicina:                    | 250 µg/ml   |
| Lincomicina                     | 150 µg/ml   |
| Espectinomicina:                | 300 µg/ml   |

(Tabla 3 y siguiente párrafo, patente de EUA 7,820,425)

5. What would be some typical parameters of this frozen thawed sperm sample?

Frozen sperm will typically be stored at  $20 \times 10^6$  sperm/straw to  $40 \times 10^6$  sperm/ml in a .5ml straw. Frozen sperm can be expected to have less than 65% motility and greater than 35% dead. Some literature suggests that, in the best case scenario, freezing then thawing sperm will kill just less than 50% of the sperm. (Page 35, Vishwanath, 2002).

6. Describe the difficulties in staining a frozen thawed sample.

5. ¿Cuáles serían algunos parámetros típicos de esta muestra congelada de espermatozoides líquido?

El espermatozoides congelado se almacenará típicamente a  $20 \times 10^6$  espermatozoides/bombilla hasta  $40 \times 10^6$  espermatozoides/ml en una bombilla de .5ml. Se puede esperar que el espermatozoides congelado tenga menos de 65% de movilidad y más de 35% muerto. Alguna literatura sugiere que, en el escenario del mejor caso, al congelar y luego derretir el espermatozoides matará solamente menos de 50% del espermatozoides (página 35, Vishwanath, 2002).

6. Describa las dificultades al teñir una muestra líquida congelada.

You expect glycerol and egg yolk in a frozen thawed sperm sample to have a negative impact on staining, and particularly on the uniformity of staining and on the viability of the sperm sample.

The constituents of egg yolk make staining difficult by reducing the effectiveness with which dye associates with the nuclear DNA of the sperm. This results in a less even staining, or might require a longer incubation period. This is substantiated by high volume ejaculates and low concentration, in these circumstance proteins in the seminal plasma are affecting the staining procedure.

Further, staining is a harmful destructive process for sperm because it requires exposing sperm to elevated temperatures and elevated pHs, both of which are known to damage sperm, which already has a limited shelf life. As such, there is a limited number of staining parameters which can be modified before a sperm sample is damaged to the point it loses its viability.

Generally, glycerol is only recommended for sperm which is to be frozen because it can generally have a negative impact on sperm viability (Parks, 1998). This damage could be exacerbated in a staining process where sperm is incubated at elevated temperatures and pHs in the presence of glycerol. Parks notes that glycerol is generally only added to chilled sperm because of the increased potential for damage at higher temperatures.

7. What difficulties would you expect in attempting to sort a stained frozen thawed sperm sample by the procedure described in US Patent 6,149,867?

The process for sorting sperm is accomplished by FACS (Fluorescence Activated Cell Sorting),

Se espera que el glicerol y la yema de huevo en una muestra de esperma líquido congelado tengan un impacto negativo en la tinción, y particularmente en la uniformidad de la tinción y en la viabilidad de la muestra de esperma.

Los constituyentes de la yema de huevo hacen difícil la tinción al reducir la eficiencia con la cual el colorante se asocia con el ADN nuclear del esperma. Esto resulta en una tinción menos uniforme, o podría requerir un periodo de incubación más prolongado. Esto se soporta en eyaculaciones de alto volumen y baja concentración, en estas circunstancias las proteínas en el plasma seminal están afectando el procedimiento de tinción.

Además, la tinción es un proceso destructivo dañino para el esperma porque requiere exponer al esperma a temperaturas elevadas y pHs elevados, ambos de los cuales se sabe que dañan el esperma, el cual ya tiene una vida de anaquel limitada. Como tal, hay un número limitado de parámetros de tinción que se pueden modificar antes de que se dañe una muestra de esperma hasta el punto de que pierda su viabilidad.

Generalmente, el glicerol se recomienda solamente para el esperma el cual se va a congelar debido a que puede tener generalmente un impacto negativo en la viabilidad del esperma (Parks, 1998). Este daño podría exacerbarse en un proceso de tinción donde el esperma se incubaba a temperaturas y pHs elevados en la presencia de glicerol. Parks señala que el glicerol se agrega solo generalmente al esperma helado debido al potencial creciente de daño a temperaturas superiores.

7. ¿Qué dificultades esperaría usted en un intento por clasificar la muestra de esperma líquido congelado por el procedimiento descrito en la patente de E.U. 6,149,867?

El proceso para clasificar el esperma se efectúa por FACS (Clasificación de Células Activadas por Fluorescencia),

which requires precise measurements of emitted fluorescence in response to laser excitation to differentiate a 3.8% difference in the amount of nuclear DNA in Y-chromosome bearing sperm and Y-chromosome bearing sperm, in bovine for example. To differentiate such small differences in nuclear DNA, the fluorescence detection must be a precise measure of DNA content in the sperm. If a sperm sample is not uniformly stained, it becomes increasingly difficult to differentiate the minor DNA differences. So, for the reasons set forth above regarding staining, it was believed egg yolk would contribute to less even staining and glycerol would increase sperm damage, contributing to difficulties in sorting.

Further, the starting concentration of frozen sperm ( $\sim 40 \times 10^6$  sperm/ml) is less than starting concentration of sperm indicated at Col. 13, lines 33-40 of Siedel ( $100 \times 10^6$  sperm/ml) and in Example 1 ( $75 \times 10^6$  sperm/ml). A more widely held current practice would be to process sperm at concentrations  $\sim 150 \times 10^6$  sperm/ml. Siedel's concentration is not only very low, but typically frozen sperm is contained within a .5 ml straw, so the total number of sperm cells in a frozen straw would be  $\sim 20 \times 10^6$  sperm.

Compounded with the fact much of the sperm will be dead when thawed (Vishwanath, 2002), a thawed sperm sample from a frozen straw provides sperm which may net only a very small number sorted viable sperm cells. Given that of the viable, evenly stained sperm cells, only  $\frac{1}{2}$  will be of any particular selected sex, the number of sorted sperm becomes even smaller, and may not provide enough sperm for artificial insemination.

lo que requiere de mediciones precisas de la fluorescencia emitida en respuesta a la excitación láser para distinguir una diferencia de 3.8% en la cantidad del ADN nuclear en el esperma que lleva el cromosoma Y, y el esperma que lleva el cromosoma Y en bovinos por ejemplo. Para distinguir tales diferencias pequeñas en el ADN nuclear, la detección de fluorescencia debe ser una medida precisa del contenido de ADN en el esperma. Si una muestra de esperma no se tiñe uniformemente, se vuelve crecientemente difícil distinguir las diferencias menores del ADN. Entonces por las razones arriba establecidas referentes a la tinción, se creía que la yema de huevo contribuiría a incluso menos tinción y el glicerol incrementaría el daño en el esperma, contribuyendo a dificultades en la clasificación.

Además, la concentración de inicio del esperma congelado ( $\sim 40 \times 10^6$  esperma/ml) es menor que la concentración de partida del esperma indicado en la Col. 13, líneas 33-40 de Siedel ( $100 \times 10^6$  esperma/ml) y en el Ejemplo 1 ( $75 \times 10^6$  esperma/ml). Una práctica actual más ampliamente mantenida sería procesar el esperma a concentraciones  $\sim 150 \times 10^6$  esperma/ml. La concentración de Siedel no solamente es muy baja, sino que típicamente el esperma congelado está contenido dentro de una bombilla de .5 ml, de manera que el número total de células de esperma en una bombilla congelada sería  $\sim 20 \times 10^6$  esperma.

Al combinarse con el hecho de que mucho del esperma estará muerto cuando se hace líquido (Vishwanath, 2002), una muestra líquida de esperma de una bombilla congelada proporciona esperma que solamente puede atrapar un número muy reducido de células clasificadas de esperma viable. Dado que de las células de esperma teñidas uniformemente, viables, solamente la mitad será de un sexo seleccionado en particular, el número de esperma seleccionado se hace incluso más pequeño, y puede no suministrar suficiente esperma para la inseminación artificial.

8. Prior to Nov. 2000, would you have thought frozen thawed sperm could be sorted by the process described in US Patent 6,149,867?

**For a combination of the reasons provided in 6, I would not have thought it was possible.**

I hereby declare that all answers provided herein of my own knowledge are true, and that all statements made on information and belief are believed to be true.

*Richard A. Lenz*  
Rick Lenz, Scientist Inquran, LLC

**Rick Lenz, Scientist Inguran, LLC**

8-16-11  
Date

8. Previo a Nov. 2000, ¿habría pensado que el esperma líquido congelado podría seleccionarse por el proceso descrito en la patente de E.U. 6,149,867?

Para una combinación de las razones suministradas en 6, no habría pensado que fuera posible.

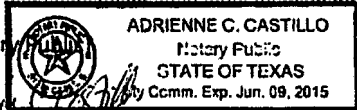
Por la presente declare que todas las respuestas aquí proporcionadas de mi propio conocimiento son verdaderas, y que todas las declaraciones hechas sobre la información y creencia se considera que son verdaderas.

**Rick Lenz, Científico Inguran, LLC**

**Fecha**

State of TEXAS County of Brazos  
Before me personally appeared said **RICK LENZ**  
and acknowledged the foregoing instrument to  
be his free act and deed this 16<sup>th</sup> day of  
August, 2011.

SEAL (Notar



Notary Public in and for the State of TEXAS  
My Commission Expires: June 29, 2015

Estado de TEXAS Condado de Brazos  
 Comparecí ante mí personalmente dicho **RICK  
 LENZ** y reconozco que el instrumento anterior es  
 de su libre acción y certifico este día \_\_\_\_\_ de  
 \_\_\_\_\_, 20\_\_\_\_\_.

**SELLO (Notario Público)**

**Notario Público en y para el Estado de TEXAS**  
**Mi Encargo termina en:** \_\_\_\_\_.



**The State of Texas**  
**Secretary of State**

**Requested for use in COLOMBIA**

**Not for use within the United States of America**

**This Apostille only certifies the signature, the capacity of the signer and the seal or stamp it bears. It does not certify the content of the document for which it was issued.**

**APOSTILLE**

**(Convention de La Haye du 5 Octobre 1961)**

|                                       |                                                                                                 |
|---------------------------------------|-------------------------------------------------------------------------------------------------|
| <b>1. Country</b>                     | <b>United States of America</b>                                                                 |
| <b>This public document</b>           |                                                                                                 |
| <b>2. has been signed by</b>          | <b>ADRIENNE C CASTILLO</b>                                                                      |
| <b>3. acting in the capacity of</b>   | <b>Notary Public, State of Texas</b>                                                            |
| <b>4. and bears the seal/stamp of</b> | <b>ADRIENNE C CASTILLO,<br/>Notary Public, State of Texas,<br/>Commission Expires: 06-09-15</b> |

**CERTIFIED**

|                                              |                              |
|----------------------------------------------|------------------------------|
| <b>5. at Austin, Texas</b>                   | <b>6. on August 17, 2011</b> |
| <b>7. by the Secretary of State of Texas</b> |                              |
| <b>8. Certificate No. N-812491</b>           |                              |
| <b>9. Seal</b>                               | <b>10. Signature:</b>        |



**Hope Andrade**  
**Secretary of State**

**ST/eg**

**DECLARATION OF RICK LENZ**

Sir:

I, Rick Lenz, hereby declare as follows:

1. I am employee of Inguran, LLC, a technology company based in the United States, operating in the field of animal reproductive biology, including the provision of commercial services and products involving the sex sorting of sperm.
2. I have extensive training and knowledge in the field of animal reproductive biology, and the provision of services related to sex sorting sperm. I am co-author on numerous research publications in the field.
3. I am aware of the current invention disclosure as described in Colombian Patent Application No. 03054828, which is a national stage application claiming priority to International Patent Application No. PCT/US01/45023 filed November 29, 2001, and published as WO 02/43574.
4. I am aware of the disclosed technology as disclosed in the cited reference, US Patent No. 6,149,867 by inventor Seidel.
5. I compared the process invention as claimed in the current application to the process as disclosed in the Seidel patent, and find that the composition of the freshly collected semen, as used for sorting in Example 1 of the Seidel patent, is different from the composition of a frozen thawed sperm sample, as would be used for sorting in the context of the current invention.
6. In my opinion, frozen thawed sperm could not be sorted in the manner described by Seidel as a commercially viable option for the production of sex sorted sperm.

I hereby declare that all statements made herein are of my own knowledge, are true, and that all statements made on information and belief are believed to be true.

**DECLARACIÓN DE RICK LENZ**

Señor:

Yo, Rick Lenz, declaro por la presente lo siguiente:

1. Soy empleado de Inguran, LLC, una empresa de tecnología con base en Estados Unidos, que opera en el campo de la biología reproductiva animal, incluyendo la disposición de servicios y productos comerciales que involucran la selección del sexo del espermia.
2. Tengo un amplio entrenamiento y conocimiento en el campo de la biología reproductiva animal, y la disposición de los servicios relacionados con la selección del sexo del espermia. Soy co-autor de diversas publicaciones de investigación en el campo.
3. Estoy al tanto de la descripción de la invención actual como se describe en la Solicitud Colombiana de Patente No. 03054828, que es una solicitud de fase nacional que reclama prioridad de la Solicitud Internacional de Patente No. PCT/US01/45023 presentada el 29 de noviembre de 2001, y publicada como WO 02/43574.
4. Estoy al tanto de la tecnología divulgada como se describe en la referencia citada, patente de E.U. No. 6,149,867 por el inventor Seidel.
5. Comparé la invención del proceso como se reivindicó en la solicitud actual al proceso como se describe en la patente de Seidel, y encontré que la composición del semen recientemente recolectado, como se usa para selección en el Ejemplo 1 de la patente de Seidel, es diferente de la composición de una muestra de espermia congelado derretido, como se usaría para seleccionar en el contexto de la invención actual.
6. En mi opinión, el espermia congelado derretido no podía seleccionarse en la forma descrita por Seidel como una opción comercialmente viable para la producción de espermia seleccionado por sexo.

Por la presente declaro que todas las afirmaciones hechas en el presente son de mi propio conocimiento, son verdaderas, y que todas las declaraciones hechas sobre información y creencia se consideran que son verdaderas.

*Rick Lenz*  
Rick Lenz, Scientist Inguran, LLC

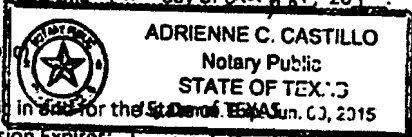
8-16-11  
Date

\_\_\_\_\_  
Rick Lenz, Cientifico Inguran, LLC

\_\_\_\_\_  
Fecha

State of TEXAS County of Brazos  
Before me personally appeared said RICK LENZ  
and acknowledged the foregoing instrument to be his  
free act and deed this 16<sup>th</sup> day of August, 2011.

SEAL (Notary Public)



Notary Public in and for the State of TEXAS  
My Commission Expires: June 24, 2015

*Adrienne C. Castillo*

Estado de TEXAS Condado de Brazos  
Ante mí compareció personalmente dicho RICK LENZ  
y reconoció que el instrument anterior es de su acción  
libre y lo subscribe este día  
\_\_\_\_\_ de \_\_\_\_\_, de 20\_\_\_\_.

SELLO (Notario Público)

Notario Público en y para el Estado de TEXAS  
Mi Encargo concluye en: \_\_\_\_\_

**ANEXO 3. ARTÍCULO “STORAGE OF BOVINE SEMEN IN LIQUID AND FROZEN  
STATE” R. VISHWANATH Y P. SHANNON.**



## Storage of bovine semen in liquid and frozen state

R. Vishwanath\*, P. Shannon

*Livestock Improvement Corporation, Private Bag 3016, Hamilton, New Zealand*

### Abstract

This review describes the historical and current methods used for storage of bovine semen. The essential physiological differences between liquid and frozen semen, their relative advantages and disadvantages are addressed, and the current state of technology, the procedures used, their merits and future possibilities are also discussed. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Liquid semen; Spermatozoa numbers; Frozen bovine semen

### 1. Introduction

Genetic advancement in the cattle population, particularly in the dairy sector has relied on two processes, namely, the use of bulls of high genetic merit and the selective rearing of calves of high breeding merit as replacements. Artificial insemination has remained the main vehicle for the rapid dissemination of valuable genes and the method of choice for dairy farmers worldwide to improve the genetic quality of their stock. This steady level of genetic progress in dairy cattle is primarily due to advances in semen technology. For the purposes of this review, the reference will be mostly with the dairy industry, for this is where technical advancement in semen technology has been captured most successfully (Chupin and Schuh, 1993; Chupin and Thibier, 1995; Cunningham, 1998; Foote, 1998).

The potential genetic contribution of a sire is described by Foote (1998) as:

$$\text{Contribution of a sire to genetic improvement} = \text{Number of progeny per sire} \times \text{Genetic superiority of the sire.}$$

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The number of progeny per sire will be determined by: (i) total sperm output of the bull; (ii) the number of sperm used per insemination; and (iii) the percentage of cows calving to a single insemination. This can be represented as:

$$\begin{aligned} &\text{Number of progeny per sire} \\ &= (\text{Number of sperm per sire} / \text{number of sperm inseminated per cow}) \\ &\quad \times \text{Proportion of cows calving to a single insemination.} \end{aligned}$$

These principles effectively determine the number of bulls required for a dairy cow population. The equation becomes particularly attractive when very few bulls are needed to service a large population of dairy cows thereby significantly raising the selection intensity (Shannon, 1978).

The requirement of semen from bulls of high genetic merit has been the main impetus for developing and refining storage technologies for cattle semen. In a 1980 survey, it was believed that the total number of inseminations worldwide exceeded 130 million (Bonadonna and Succi, 1980). A more recent survey (Chupin and Thibier, 1995) showed that the total number of doses of semen produced exceeded 200 million, with more than 95% of this processed as frozen product. About 4 million doses were used as liquid semen, and this was restricted primarily to New Zealand with smaller amounts in France, The Netherlands, Australia and Eastern Europe. In this review, the main focus will be on the well-known storage technologies, which include liquid and frozen stored semen along with a brief discussion on emerging technologies for semen storage in cattle.

## 2. Liquid storage of semen

There have been many reports since the turn of the century on diluting media for semen of livestock with much of this work originating in the former Soviet Union (Anderson, 1945). Each diluent, ranging from a simple salt solution to the more complex buffered medium had its own merits. Perhaps the accepted principle of semen dilution technology was that survival of spermatozoa for extended periods was inversely related to their metabolic activity. To be useful for artificial insemination, diluted semen had to have a minimum shelf-life of between 2 and 4 days to provide for easy transport and use in distant locations. It was this guiding principle that led to the initial storage temperature of 5°C (Salisbury and Van Demark, 1961). The predominant effect of storage at 5°C was a lowering of metabolic rate of spermatozoa, which contributed to extended survival. The discovery that egg yolk was a useful additive in increasing the preserving properties of the various media, added further impetus to this work (Phillips, 1939). Many extenders have been developed for liquid storage of semen, and this chapter only provides a brief description of some of the major developments. Detailed discussions on some of the early diluents are available in a comprehensive review by Roote (1978).

### 2.1. Diluents for storage of semen at refrigerated temperatures

The early diluents for storage at refrigerated temperatures were significantly influenced by the discovery of egg yolk as a useful additive, and the primary buffering

Table 1

Comparison of 60- to 90-day non-returns (% NRR) to inseminations with bovine semen diluted to 8 million spermatozoa/ml in CUE and Tris media. Data from Foote (1978). Diluent compositions are shown in Table 2

| Diluent | No. 1st inseminations | % NRR |
|---------|-----------------------|-------|
| CUE     | 5981                  | 73.0  |
| Tris    | 5673                  | 73.3  |

component in these diluents was phosphate (Phillips, 1939). Subsequently, citrate was found to have adequate buffering capacity with an enhanced period of survival of spermatozoa stored at 5°C (Willett and Salisbury, 1942). Citrate then became the salt of choice, as its chelating properties improved the solubility of protein fractions in egg yolk. Egg yolk has been a more common additive, but homogenised whole milk, fresh or reconstituted skim milk and coconut milk have also been used to preserve fertility of bovine spermatozoa (Melrose, 1962; Norman et al., 1962).

Many of the zwitterionic buffers (Good et al., 1966) provided good buffering capacity over a wide pH range. Tris, TES, MES, HEPES, PIPES, MOPS and BES titrated with hydrochloric acid were tested at various pH levels as semen diluents and all of them were similar in suitability (Foote, 1972) cited by Foote (1978). However, the Tris-based diluent has become more universal, and this buffering medium combined with egg yolk and glycerol has been tested extensively (Davis et al., 1963a,b). Motility of spermatozoa was slightly lower in Tris medium buffered to lower pH (6.25 vs. 6.75) than in Cornell University Extender (CUE), but good results were obtained in a fertility trial with both media (Table 1).

A further field trial demonstrated that a lower pH (6.5) and the presence of glycerol in Tris medium improved fertility, compared with higher pH and absence of glycerol (Foote, 1970). The results were comparable with semen diluted with CUE. The conclusion from these series of studies was that the Tris diluent was suitable for storage of semen at refrigerated or ambient temperature and also in frozen state. The diluents shown in Table 2, and some subsequent modifications to these have been used in field trials with varying success (Bartlett and Van Demark, 1962; Van Demark and Bartlett, 1962; Shannon, 1965, 1978; Foote, 1978). In the case of the Illini Variable Temperature (IVT) diluent, the main changes have been in the ratio of bicarbonate to citrate and the concentration of glucose increasing from 0.3% to 1.2%.

#### 2.1.1. Milk-based diluents

The historical origin of milk as a diluent for bull semen has been described by Salisbury and Van Demark (1961). The first report on the use of milk originated in Germany (Koelliker, 1856) cited by Salisbury and Van Demark (1961). This observation went unnoticed until Underbjerg et al. (1942) compared fertility of bull semen stored in egg yolk-phosphate and autoclaved milk diluents. The results were similar for both media. The methods of preparation of milk diluents and the various combinations have been reviewed by Melrose (1962) and Foote (1978). The important aspect of storage of semen in milk-based extenders was the necessity to inactivate the lactenin (a toxic

Table 2  
Composition of diluents for storage of bovine semen at low (5°C) and ambient temperatures. Ingredients are in g/100 ml of medium

| Ingredients                  | Egg yolk–phosphate<br>(Phillips, 1939) | Egg yolk–citrate<br>(Salisbury et al., 1941) | Original IVT diluent<br>(Van Demark et al., 1957) | CUE<br>(Foote et al., 1960) | Tris medium for<br>ambient storage<br>(Foote, 1970) | CAPROGEN®<br>(Shannon, 1965) |
|------------------------------|----------------------------------------|----------------------------------------------|---------------------------------------------------|-----------------------------|-----------------------------------------------------|------------------------------|
| Temperature of storage       | 5°C                                    | 5°C                                          | 5°C                                               | 5°C and ambient             | 5°C and ambient                                     | Ambient                      |
| Tris                         |                                        |                                              |                                                   |                             | 3.028                                               |                              |
| Potassium hydrogen phosphate | 0.2                                    |                                              |                                                   |                             |                                                     |                              |
| Sodium hydrogen phosphate    | 2                                      |                                              |                                                   |                             |                                                     |                              |
| Sodium citrate               |                                        | 3.6                                          | 2                                                 | 1.45                        |                                                     | 2                            |
| Sodium bicarbonate           |                                        |                                              | 0.21                                              | 0.21                        |                                                     |                              |
| Potassium chloride           |                                        |                                              | 0.04                                              | 0.04                        |                                                     |                              |
| Glucose                      |                                        |                                              | 0.3                                               | 0.3                         | 1.25                                                | 0.3                          |
| Citric acid                  |                                        |                                              |                                                   | 0.087                       | 1.675                                               | 1                            |
| Glycine                      |                                        |                                              |                                                   | 20                          |                                                     | 0.014                        |
| Glycerol                     |                                        |                                              |                                                   |                             | 8                                                   | 1.25                         |
| Catalase                     |                                        |                                              |                                                   |                             |                                                     | 0.003                        |
| Caproic acid                 |                                        |                                              |                                                   |                             |                                                     | 0.025                        |
| Egg yolk (%)                 | 50                                     | 50                                           | 10                                                | 20                          | 25                                                  | 5                            |
| Antibiotics                  |                                        |                                              | Yes                                               | Yes                         | Yes                                                 | Yes                          |
| Gas phase                    |                                        |                                              | CO <sub>2</sub>                                   | self carbonating            |                                                     | N <sub>2</sub>               |

factor) by heating. Different preparations of milk needed different heating requirements to selectively inactivate the lactenin, but still maintain the integrity of the protein and sugar moieties in milk. Glycerol added to the milk-based diluents was advantageous and maintained fertility of bull spermatozoa for 4 days (O'Connor and Smith, 1959; Almquist, 1962). In general, milk-based extenders have been successful in maintaining fertility and gave comparable results to egg yolk–citrate diluents (Foote, 1978). Egg yolk (5–10%) added to milk-based extenders seemed to inactivate the toxic factor in milk. This process has overcome the problem of heating the skim or fortified milk before addition to semen, and also adequately maintained the fertility of diluted bovine semen stored at 5°C.

## 2.2. Storage of semen at ambient temperature

It has been the maxim of semen dilution technology that survival of spermatozoa in diluted state is inversely related to their metabolic activity. The pursuit of this principle led to the development of egg yolk diluents for storage of semen at 5°C (Phillips, 1939) and deep freeze techniques for total immobilisation of spermatozoa at very low temperatures (Polge et al., 1949; Polge and Rowson, 1952). In order to store semen at temperatures above 5°C and achieve satisfactory results, alternative methods of metabolic inhibition were attempted. Thus, Van Demark and Sharma (1957), proposed CO<sub>2</sub> narcosis, Norman et al. (1958) suggested lowering the pH and Shannon (1965) proposed N<sub>2</sub> gassing as methods to inhibit metabolic activity of spermatozoa.

Storage at 5°C reduces metabolic activity, but not all changes associated with lower temperatures are beneficial to spermatozoa. For example, the activity of the Na<sup>+</sup>/K<sup>+</sup> pump decreases with reduced temperatures such as 5°C, but is unable to cope with diffusion of ions across the cell membrane (Quinn and White, 1966, 1967; Sweadner and Goldin, 1980). A consequent increase in the intracellular concentration of Na<sup>+</sup> is detrimental to survival of spermatozoa (Makler et al., 1981). It was then postulated that storage at ambient temperature may be superior to storage at 5°C, provided the medium in which spermatozoa are suspended inhibits those pathways that are detrimental to their survival at higher temperatures (Shannon and Curson, 1972b, 1982a, 1984). The temperature of storage is an important consideration. The optimum temperature range is considered to be 18°C to 24°C (Shannon and Curson, 1984). Storage at temperatures above this results in lower fertility, compared to storage at 5°C (Foote and Bratton, 1960; Bartlett and Van Demark, 1962).

Another significant development in ambient temperature diluent technology has been the change in the level of egg yolk in the diluent. The ratio of egg yolk to buffer became particularly important at higher storage temperatures than it was with storage at 5°C (Foote and Bratton, 1960; Shannon and Curson, 1983). Historically, semen diluents have included anywhere from 12.5% to 50% egg yolk in the medium. Efforts to decrease the egg yolk concentration in the simple phosphate and citrate buffers gave no advantage in survival of spermatozoa or fertility (Foote and Bratton, 1960). Egg yolk does protect the sperm cells from the toxic effects of seminal plasma; however, it also provides substrates (aromatic amino acids such as L-phenylalanine) for H<sub>2</sub>O<sub>2</sub> production by an aromatic amino acid oxidase (AAAO) released from dead cells to the detriment of live

Table 3

Percentage of NRRs (49 day) for semen diluted in CAPROGEN® media containing differing levels of egg yolk. Data from Shannon and Curson (1983)

| Trial | Egg yolk (%) |            |            |            |
|-------|--------------|------------|------------|------------|
|       | 20           | 5          | 2          | 1          |
| 1     | 66.5 ± 0.4   | 67.6 ± 0.4 |            |            |
| 2     |              | 67.4 ± 0.3 | 67.9 ± 0.3 |            |
| 3     |              | 67.6 ± 0.7 | 67.4 ± 0.7 | 66.1 ± 0.7 |

spermatozoa (Shannon and Curson, 1972b, 1982b). The amount of egg yolk required in semen diluents to provide protection against seminal plasma toxins is proportional to the amount of seminal plasma in diluted semen (Shannon and Curson, 1972a). Thus, when semen is diluted to a high rate, and the seminal plasma concentration is consequently reduced, there would be some advantage in reducing the egg yolk concentration. This was borne out in a large fertility trial, where a decrease in egg yolk concentration from 20% to 5% had no detrimental effect on fertility. Further decrease of egg yolk concentration affected fertility of some sires, suggesting that the level of egg yolk was insufficient in some cases to be completely protective (Table 3).

#### 2.2.1. Coconut milk extender

Coconut milk extenders have been equivalent to skim milk–glycerol, CUE or CAPROGEN® in maintaining motility and survival of spermatozoa (Norman and Rao, 1972; Foote, 1978). This extender is quite simple and contains 15% decanted coconut milk boiled for 10 min, 2.2% sodium citrate, antibiotics and 5% egg yolk. The presence of egg yolk was essential to provide a lipid component to the medium. In some cases, the medium has been supplemented with 0.1% calcium carbonate (Norman et al., 1958). A recent report on the use of Coconut extract and Coconut milk on ram semen showed dramatic maintenance of motility over a 48 h period of 30°C but no fertility trials have been reported (Chairussyuhur et al., 1993). There have been no recent reports on significant advances in this front in the literature.

#### 2.2.2. Immobilisation of spermatozoa by low pH

An early observation in 1924 by Krshyshkovsky and Pavlov, cited by Norman et al. (1958), showed that spermatozoa were immobilised when placed in sealed tubes at room temperature, but subsequent exposure to air produced a resumption of activity. Inhibition of sperm motility was due to the decrease of pH by the accumulation of lactic acid in the medium. Further studies by Norman et al. (1958) confirmed the finding and suggested the decrease of pH as an effective method to inhibit metabolic activity of spermatozoa. In this study, conclusive evidence was obtained that lowering the pH substantially reduced metabolic activity measured by O<sub>2</sub> consumption, lactate production, and motility of live spermatozoa. This effect was reversible, as activity resumed when the old diluting medium was replaced after 150 h incubation with fresh medium at neutral pH. By merely altering the pH from 5.76 to 7.45, motile activity of spermatozoa could

be altered from relative senescence to rapid movement (Norman et al., 1958). The effect of acidic conditions on sperm metabolism was identified by Koellikker (1856), cited by Norman et al. (1958) and Guenther (1907), who concluded that activity of spermatozoa is a function of hydrogen ion concentration. A decrease in pH down to 5.5 was well tolerated by spermatozoa, and the effect could be reversed by alkaline conditions, but a pH below 5.5 was spermicidal and caused irreversible enzyme denaturation.

2.2.3. Immobilisation of spermatozoa in diluents gassed with carbon dioxide

Carbon dioxide was found to be a very effective inhibitor of sperm motility (Shettles, 1940). Early experiments showed that sperm motility was reversibly inhibited when exposed to CO<sub>2</sub> for short periods, but prolonged exposure to this gas proved toxic to sperm cells. Van Demark and Sharma, 1957 proposed CO<sub>2</sub> narcosis as an effective means to maintain viability and fertilising ability of bovine spermatozoa for 6 to 7 days at room temperatures. The first diluent designed on the basis of CO<sub>2</sub> immobilisation of spermatozoa was the IVT diluent (Van Demark et al., 1957). The IVT diluent contained a mixture of salts, sugar and antibacterial agents, and was saturated with CO<sub>2</sub> until the pH decreased to 6.35. The final mixture contained 10% egg yolk. Bovine semen extended with this diluent and stored at room temperature maintained fertility for over 3 days (Bartlett and Van Demark, 1962). Several diluents were then formulated using this concept to optimise sperm survival and to extend the shelf-life of diluted semen (Table 2). By optimising the concentration of bicarbonate in the medium (0.1 M), and at higher concentrations of glucose (0.067 M) in the IVT diluent, sperm motility was maintained at high levels (> 45%) for over 90 days at 5°C. While motility was maintained for extended periods, fertility decreased sharply after 2 days of storage at 5°C, (Bartlett and Van Demark, 1962). This inconsistency in fertility result was evident also in other studies, where CO<sub>2</sub> narcosis was used to maintain motility and fertility of spermatozoa for extended periods (Dunn and Foote, 1958; McFee et al., 1958; Foote et al., 1960). The main difference between the IVT diluent and the CUE was that CUE was self carbonating and relied on the action of citric acid on bicarbonate to release CO<sub>2</sub> with no major effect on the pH of the medium (Table 2). The CUE was a substantial improvement on the IVT diluent in this regard. Numerous field trials have confirmed that CUE along with CAPROGEN® are the most successful liquid semen diluents to date (Table 4; Shannon, 1964; Foote, 1978).

Table 4  
Fertility of bull spermatozoa stored in CUE and CAPROGEN® diluents. Data from Shannon (1964) and expressed as % NRR at 49 days

| Diluent              | Day of collection    |       | Day after collection |       |
|----------------------|----------------------|-------|----------------------|-------|
|                      | No. of inseminations | % NRR | No. of inseminations | % NRR |
| CUE                  | 59,091               | 62.8  | 25,309               | 65.2  |
| CUE + glycerol       | 30,058               | 63.1  | 13,909               | 64.9  |
| CAPROGEN®            | 73,993               | 62.8  | 37,758               | 63.5* |
| CAPROGEN® + glycerol | 35,351               | 62.7  | 17,957               | 64.9  |

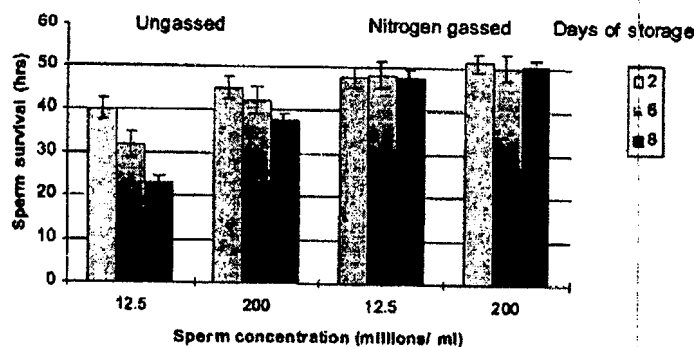


Fig. 1. Incubated life of spermatozoa (h) stored at 12.5 million/ml and 200 million/ml at 5°C. The samples were incubated at 37°C at 12.5 million sperm/ml. Data from Shannon (1965).

#### 2.2.4. Metabolic inhibition by nitrogen gassing

Shannon (1964, 1965) developed the CAPROGEN® diluent for bovine semen, which replaced the self-carbonating concept with an ingenious method to reduce the dissolved O<sub>2</sub> levels in the medium with N<sub>2</sub> gas. This had no effect on the pH, but substantially reduced the metabolic activity of spermatozoa. An additional effect of N<sub>2</sub> gassing was that it nullified the adverse effect of high dilution on sperm survival. Nitrogen saturation halted the decline in incubation life of stored spermatozoa. This effect was more noticeable when spermatozoa were stored in a dilute form (12.5 million/ml), compared to storage in a concentrated state (over 200 million/ml, Fig. 1). At the same time, inclusion of volatile saturated fatty acids (Shannon, 1962) and catalase (Shannon, 1972, 1973; Macmillan et al., 1978) significantly improved the length of time that diluted bovine semen could be stored. The diluent was originally developed for use at 5°C, but proved superior for semen stored at temperatures between 15°C and 27°C (Shannon and Curson, 1984). The conception rates with semen stored in CAPROGEN® were 0.9% better at the elevated temperatures on the day of collection and 2.3% higher on the following day compared to semen stored at 5°C (Table 5; Shannon, 1971).

#### 2.3. Requirements for storage of bull semen at ambient temperatures

Storage of semen at 5°C was not always easy or convenient, therefore, methods to store semen at ambient or room temperatures were actively investigated. The require-

Table 5

Fertility of semen diluted and stored in CAPROGEN® at 5°C or ambient temperature (18–24°C) and used on day of or day after collection. Data from Shannon (1971). Ejaculates were split between the two treatments and fertility measured as % NRR at 49 days

| Storage times | Storage at 5°C       |       | Ambient temperature  |       |
|---------------|----------------------|-------|----------------------|-------|
|               | No. of inseminations | % NRR | No. of inseminations | % NRR |
| 6–12 h        | 268,201              | 65.3  | 44,354               | 66.2  |
| 12–24 h       | 172,663              | 66.6  | 29,772               | 68.9* |

\* Significantly different to storage at 5°C ( $p < 0.05$ ).

ments regarding diluting media that could accommodate the decrease in metabolic activity of spermatozoa when stored at ambient temperature are the following.

(i) An energy source in the form of simple sugars (glucose, fructose). Bovine spermatozoa function adequately under both aerobic and anaerobic situations and the rate of respiration can be controlled by the level of substrates (Hammerstedt et al., 1988; Krzyzosiak et al., 1999) and temperature of storage (Hammerstedt and Hay, 1980; Inskeep and Hammerstedt, 1985).

(ii) A buffered medium to accommodate pH changes, usually in the form of citrate, Tris, HEPES, MOPS, or bicarbonate buffers.

(iii) Glycerol, a dual purpose additive, to provide osmolality to the medium and as an invasive thermal protectant. In liquid semen diluents, glycerol has been shown to reduce the decline in fertility associated with the aging of spermatozoa (Shannon, 1964). In some cases, however, it did decrease fertility with semen used shortly after collection (O'Connor and Smith, 1959; Almquist, 1962). The effect of glycerol may depend on the basic diluent to which it has been added. The addition of 1% glycerol to an egg yolk–citrate–glycine diluent increased the conception rate by  $1.4 \pm 0.7\%$  for semen stored for 24 h at ambient temperature (Shannon, 1964). The beneficial effect of glycerol was also noticed when added to milk and Tris diluents with an increase in conception rates of 1.4% and 2.5%, respectively (Foote, 1970; Bratton and Foote, 1973, cited by Foote, 1978 and Stewart 1964).

(iv) Egg yolk or other macromolecular substances such as skim milk, whole milk, or coconut milk provide thermal protection to spermatozoa. The inclusion of macromolecular substances, particularly egg yolk has benefit even when sperm cells are not subjected to cold shock. Perhaps the most important effect of egg yolk is its ability to bind seminal plasma fractions that have a detrimental effect on survival of spermatozoa (Shannon and Curson, 1972a; Al-Somai et al., 1994; Prendergast et al., 1995).

(v) A combination of antibiotics to provide both bacteriostatic and bactericidal protection.

Other additives to diluents for storage of semen at ambient temperature have also influenced the keeping quality of bovine semen with some positive effects on conception rates. Some of these additives are listed below.

#### 2.3.1. Glycine

The reason why glycine has been used as an additive to semen diluents is still not clear. Many plants, unicellular organisms and marine elasmobranchs, accumulate compatible solutes, which provide protection during stress situations (Yancey and Somero, 1979; Aspinall and Paleg, 1981). Some of these compounds such as free amino acids and quaternary nitrogen containing compounds retard thermal denaturation of enzymes (Paleg et al., 1981), provide thermal protection (Heber et al., 1971) and maintain enzyme structure and function (Bowlus and Somero, 1979). Conflicting fertility results have been reported with diluents containing glycine (Strom, 1956; Stower and Bud Hasaim, 1957; Foote et al., 1958; Salisbury and Van Demark, 1961; Shannon, 1964). A fertility trial with 20% egg yolk–citrate–glucose diluent containing 1% glycine gave a fertility advantage of 2.1% on the day of collection and 2.7% with semen stored for 24 h, compared to a simple 20% egg yolk–citrate diluent (Shannon, 1964). The advantage

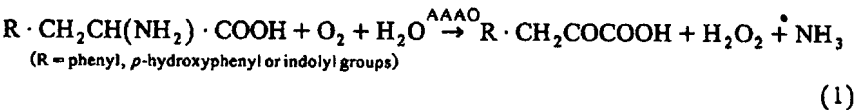
appears to be directly attributable to the glycine–glucose moiety. Presence of glycine in diluents for frozen semen has been detrimental to post-thaw survival of spermatozoa (Martin, 1965).

2.3.2. Caproic acid

Volatile fatty acids included in semen diluents is presumed to aid in maintaining membrane integrity and fluidity (Shannon, 1962). While this feature has not been investigated in detail, it is well known that lower-order fatty acids will prolong the respiratory activity of spermatozoa for extended periods (Mann and Lutwak-Mann, 1981). Caproic acid enhances cell survival and fertility of diluted bovine semen. In a large-scale fertility trial, addition of caproic acid had no significant effect on fertility with semen used on the day of collection, but a significant increase (+1.3%) was seen on the day after collection (Table 6; Shannon, 1962).

2.3.3. Catalase

The medium for dilution and storage of semen has significant effects on the integrity of spermatozoa. Within an aerobic or even a partially aerobic system, the production of reactive oxygen species is inevitable (Mann and Lutwak-Mann, 1975; Fridovich, 1978, 1981; Mann et al., 1980). The most damaging are the superoxide anion ( $O_2^-$ ), peroxide ( $H_2O_2$ ) and the hydroxyl free radical ( $OH\cdot$ ). The reactions producing these free radicals are more active when semen is stored at ambient temperatures than in frozen state. Peroxide is the more pernicious of the pro-oxidants formed. The main source of peroxide during storage of bovine semen at ambient temperature is the oxidative de-amination of aromatic amino acids by a membrane bound AAAO as shown in the Eq. (1).



The reaction was first described for bovine spermatozoa by Tosic and Walton (1946, 1950). Subsequently, Shannon and Curson (1972b) demonstrated that AAAO activity was restricted to the dead sperm population. The properties of AAAO are well established. The enzyme is heat and acid labile: it is inactive in live spermatozoa and is

Table 6  
Conception rates of semen diluted in 14G<sup>a</sup> diluent containing caproic acid (0.025%). Data from Shannon (1962) and expressed as % NRR at 49 days

| Age of semen | 14G (control)        |       | 14G + caproic acid   |                   |
|--------------|----------------------|-------|----------------------|-------------------|
|              | No. of inseminations | % NRR | No. of inseminations | % NRR             |
| 6–18 h       | 224,275              | 64.1  | 41,997               | 63.7              |
| 24–36 h      | 95,587               | 64.3  | 16,831               | 66.9 <sup>*</sup> |

<sup>a</sup>The 14G diluent is the precursor to the current CAPROGEN<sup>®</sup> medium containing all the basic ingredients except caproic acid.  
<sup>\*</sup>Significantly different to control (*p* < 0.05).

Table 7

Effect of catalase in CAPROGEN® diluent on fertility of bovine semen stored at ambient temperature and used on day of collection (6–18 h) and day after collection (24–48 h). Data from Shannon (1968) and fertility was measured as % NRR at 49 days

|         | Age of semen | CAPROGEN®            |       | CAPROGEN® + catalase |        |
|---------|--------------|----------------------|-------|----------------------|--------|
|         |              | No. of inseminations | % NRR | No. of inseminations | % NRR  |
| Trial 1 | 6–18 h       | 94,671               | 63.9  | 16,117               | 64.7   |
|         | 24–48 h      | 148,286              | 68.1  | 27,584               | 69.7 * |
| Trial 2 | 6–18 h       | 30,233               | 63.5  | 146,337              | 64.5   |
|         | 24–48 h      | 44,126               | 66.2  | 230,107              | 68.1 * |

\* Significantly different to CAPROGEN® ( $p < 0.05$ ).

released from dead cell membranes in the presence of citrate. It is confined to the tail region of bull spermatozoa and the activity of AAAO is also affected by the oxygen tension in the diluent. It was established from kinetic studies that the response of this enzyme increased with increasing temperature and duration of storage (Shannon and Curson, 1972b, 1982a,b). The enzyme is specific for L-aromatic amino acids and in particular, L-phenylalanine was significantly more toxic than L-tyrosine or L-tryptophan (Macmillan et al., 1972). Egg yolk is an excellent source of aromatic amino acids, particularly L-phenylalanine.

The levels of peroxide generated in the storage medium can be minimised by the presence of catalase (Shannon, 1968). The significance of this reaction is that in the breakdown of peroxide, half the amount of oxygen is returned back to the system (Eq. (2))



Two separate field trials substantiated the effect of catalase and the results are shown in Table 7. With extended storage times, the beneficial effect of added catalase became evident and the field trial clearly emphasises the need to either reduce or abolish endogenous peroxide production during liquid storage of semen.

#### 2.4. Factors influencing fertility after liquid storage

##### 2.4.1. The effect of duration of storage on fertility of spermatozoa

Bovine spermatozoa stored at ambient temperature in CAPROGEN® diluent show a slow decrease in motility over an extended period (about 3–4 weeks), but the concomitant decrease in fertility is significantly higher (Fig. 2). Fertility, as measured by non-return rate (NRR), is maintained for the first 3 to 5 days after dilution ( $69.9 \pm 1.2\%$ ), followed by an accelerating decrease in NRR until day 10 of storage ( $41.5 \pm 3.7\%$ ) (Vishwanath and Shannon, 1997). Bartlett and Van Demark (1962) showed a similar dichotomy in response to storage at 5°C. Semen stored in a medium containing bicarbonate, catalase and glucose and gassed with CO<sub>2</sub> had 56% motile spermatozoa after 40 days of storage. However, fertility decreased in the first few days of storage and was down on average by 8–14% by day 3 (Bartlett and Van Demark, 1962).

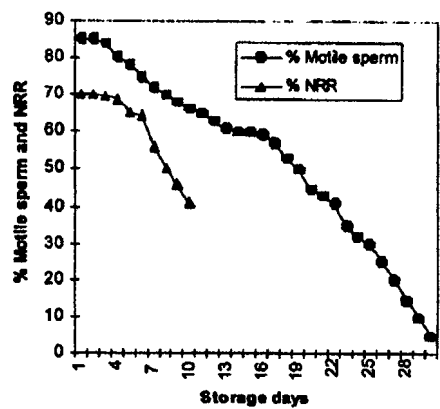


Fig. 2. The effect of ambient temperature (18–21°C) storage in CAPROGEN® on sperm motility and % NRR at 24 days. Data from Vishwanath and Shannon (1997).

The physiological reasons for this rapid decline in fertility of spermatozoa stored at ambient temperature is presumed to be due to three factors: extracellular oxidative stress, effects of seminal plasma and endogenous free radical production. The combined effects of these three factors could lead to significant damage to spermatozoa. This is perhaps the main reason why semen stored at ambient temperature is restricted in shelf-life with a declining fertilising potential. (Vishwanath and Shannon, 1997).

2.4.2. Effect of number of spermatozoa inseminated on fertility of liquid stored semen

An important factor that contributes to the fertility of diluted semen is the dose rate or absolute sperm numbers in a given inseminate. Salisbury and Van Demark (1961) have described the relationship between semen quantity (sperm numbers) and semen quality (fertilising potential). The central point in this concept is that up to a threshold level,

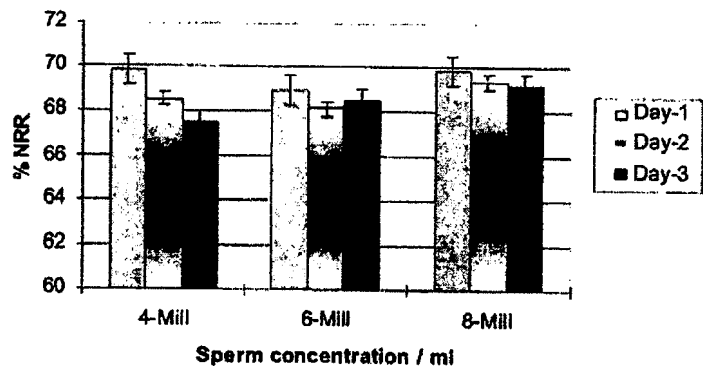


Fig. 3. NRRs (24 day) for different sperm concentrations in liquid semen inseminated on days 1–3 after collection. Day 1 is the day after collection of semen. Data from Vishwanath et al. (1996).

fertility is under the influence of sperm numbers. This threshold varies significantly between bulls. Once this level is attained, fertility is unaffected by further increases in sperm concentration. This effect has been adequately demonstrated in field trials where some of the inherent differences in the fertility of different bulls can be compensated for by altering the absolute numbers of spermatozoa in the inseminate (den Daas, 1992; Shannon and Vishwanath, 1995). The effect of sperm numbers being able to influence fertility is a defined compensable element and increasing sperm concentration does alter the probability of fertilisation (Saacke et al., 1994; Shannon and Vishwanath, 1995). However, increasing sperm numbers does not alter the rate of decrease in fertility after 5 to 6 days of storage at ambient temperature (10–21°C, Vishwanath, unpublished information). The change in NRR for different sperm concentrations in liquid semen is shown in Fig. 3. The probability of fertilisation is quite high on the first day of use at all three sperm concentrations (4, 6 and 8 million/ml), and they are not significantly different from each other. On the contrary, with storage, the NRR is lower on days 2 and 3 at the lowest sperm concentration of 4 million/ml ( $p < 0.05$ ). The reasons could be:

- probability of fertilisation decreased due to lower sperm numbers;
- effect of dilution compounding the sperm aging effect; and
- bull  $\times$  dose rate interaction.

### 3. Frozen storage of semen

Effective storage of semen in frozen state implies a complete arrest in the developmental process of sperm cells that began in the testis and continued through the epididymis and after ejaculation. The logical conclusion for this process is the presence of spermatozoa in the female tract preparing for fertilisation. Watson (1995) described the frozen preservation as “a hiatus in the process that demarcates this continuum of sperm development with a period of suspended animation that eventually leads to successful fertilisation”. There is an argument that spermatozoa stored at  $-79^{\circ}\text{C}$  (in dry ice) or at  $-196^{\circ}\text{C}$  (in liquid nitrogen) retain their fertilising potential indefinitely. Mazur (1980) proposed a time period of between 3000 and 10,000 years before genome destruction, while insemination trials with frozen stored semen (Salisbury and Hart, 1970) suggest a far shorter time period for survival at the above-mentioned low temperatures with no change in fertilising potential. However, other evidence suggests that this could have been due to inadequate maintenance of temperatures. With more advanced cryogenic vessels, it is possible to maintain spermatozoa in a frozen state for a very long period.

The fortuitous discovery of glycerol as an effective cryoprotective agent (Polge et al., 1949; Polge and Rowson, 1952) introduced a completely new system of semen storage, a method which is widely in practice today. An axiom of semen freezing is that even with the best preservation techniques and all the developments that have occurred over the years, the best cell recovery after thawing is just over 50%.

### 3.1. *Effects of freezing*

The stresses associated with freezing are primarily the temperature changes that spermatozoa are subjected to during the process of cooling, the injurious effects of the media components and cryoprotectants themselves during the process, and finally the effects of thawing. The basic principles of freezing were established quite early and several authors have reviewed the physical effects of each of the processes mentioned above (Mazur, 1963, 1965; Watson, 1979, 1995). The physical effects of cooling and then freezing a cell suspension results in a number of changes in the external environment in terms of water and solute movement. The rate at which water moves out of the cell during this process plays an important role in determining cooling rates, which have to be optimised for cell survival after thawing (Mazur, 1963, 1977). The curves for survival vs. cooling rate show a biphasic response. The optimum cooling rate is between 80°C/min and 120°C/min with faster and slower cooling rates resulting in lower survival (Watson, 1979, 1995; Woelders, 1997). This also has bearing on a period of withholding times at different temperatures to allow for equilibration before freezing. The purpose of this equilibration time at certain temperatures is to allow for the translocation of water and thereby reduce the injurious effect of ice nucleation during the freezing and thawing process.

### 3.2. *Diluents and cryoprotectants for freezing of semen*

The basic components of the diluents for freezing of semen are essentially the same as those used for ambient temperature storage. The general requirements are:

- ionic or non-ionic substances to maintain the osmolarity and to buffer the medium;
- a source of lipoprotein or high molecular weight material to prevent cold shock, such as egg yolk or milk;
- glycerol, propane-diol or DMSO to offer cryoprotection;
- glucose or fructose as an energy source; and
- other additives such as enzymes and antibiotics.

Phillips (1939) made a crucial discovery when he found that egg yolk added to the semen diluent had a beneficial effect on fertility. Phillips and Lardy (1940) recommended the use of a diluent containing equal parts of phosphate buffer and egg yolk as a protective agent against cold shock. Salisbury et al. (1941) presented conception results from field trials with bovine semen diluted in an egg yolk–sodium citrate diluent (EYC). Since then, egg yolk in combination with Tris and citrate has been the most common constituent in most freezing diluents for bovine semen. The reason why citrate is a better ionic substitute than phosphate is because phosphate inhibits the oxidation of lactic acid causing its accumulation (White, 1956). Davis et al. (1963a,b) developed a 0.2 M Tris-buffered 20% egg yolk extender (TRIS-EY). When compared with EYC and skim milk, TRIS-EY was found to be significantly better at maintaining post-thaw motility. Steinbach and Foote (1964) confirmed this observation. Field trials with cooled but unfrozen semen at sperm concentrations of 4 to 8 million /ml failed to show evidence of improved fertility with TRIS-EY compared with EYC (Foote, 1970). Compositions of these diluents and their modifications are shown in Table 8.

Table 8

Composition of Tris- and citrate-based diluents for freezing bovine semen. All diluents contain antibiotics at approximately 100,000 units of penicillin and dihydrostreptomycin/100 ml of the diluent. All values are given in g/100 ml of the medium, unless otherwise mentioned

| Diluent component        | Tris–yolk–glycerol<br>(Davis et al., 1963a) | Tris–fructose<br>yolk–glycerol<br>(Steinbach and Foote, 1964) <sup>a</sup> | Citrate–yolk–fructose–glycerol<br>(Steinbach and Foote, 1964) <sup>a</sup> | Netherlands<br>extender<br>(De Leeuw et al., 1993) |
|--------------------------|---------------------------------------------|----------------------------------------------------------------------------|----------------------------------------------------------------------------|----------------------------------------------------|
| Tris <sup>b</sup>        | 3.03                                        | 2.4                                                                        |                                                                            | 3.15 <sup>c</sup>                                  |
| Glycerol                 | 14 ml                                       | 8.8 ml                                                                     | 7.2 ml                                                                     | 6 ml                                               |
| Fructose                 |                                             | 1                                                                          | 1                                                                          | 1                                                  |
| Citric acid monohydrate  | 1.69                                        | 1.354                                                                      |                                                                            | 1.23                                               |
| Sodium citrate dihydrate |                                             |                                                                            | 2.32                                                                       |                                                    |
| Egg yolk                 | 20 ml                                       | 20 ml                                                                      | 20 ml                                                                      | 20 ml                                              |

<sup>a</sup>Original diluent modified by Foote (1970) to include fructose.

<sup>b</sup>Tris (hydroxymethyl) aminomethane.

<sup>c</sup>Tris (hydroxymethyl) aminomethane hydrochloride.

### 3.2.1. Egg yolk

There have been many attempts to find the protective component in egg yolk with the aim to prepare a 'chemically defined diluent', and thus reduce the possibility of transmission of harmful pathogens, the likely inconsistencies between different batches of egg yolk and the yolk globules which interfere with microscopic examination of diluted semen. Early experiments on the protective action of egg yolk soon led to research in the specific components of egg yolk that might be responsible for the cryoprotective action, namely, phosphatidylcholine (lecithin), phospholipids, lipid extracts, lipoprotein fractions and specific lipoproteins (Mayer and Lesley, 1945; Blackshaw, 1954; Blackshaw and Salisbury, 1957; Martin, 1963; Lanz et al., 1965; Gebauer et al., 1970). There was a consensus among these studies that lecithin provides some protection to spermatozoa during cold shock and freezing. One of the major problems with lecithin is that it is insoluble in aqueous solutions, forming an unstable suspension. It partly precipitates in the absence of vigorous stirring and can break down to form lysolecithin which is toxic to spermatozoa (Jones, 1976). Synthetic liposomes made up of dioleoyl phosphatidyl choline, phosphatidyl choline, phosphatidyl serine and combinations thereof with cholesterol were effective in preventing cooling damage to spermatozoa, but were a poor substitute compared to egg yolk in preventing freeze–thaw damage (De Leeuw et al., 1993).

The major component of egg yolk offering protection is a phospholipid moiety of a low density lipoprotein fraction (Pace and Graham, 1974; Foulkes, 1977; Watson, 1981). Further analysis of the type of lipoprotein extracted from whole egg yolk by water, saline and citrate revealed that the major extract is a cationic lipoprotein fraction that preferentially bound to the sperm membrane and afford far greater protection. This protection is assumed to result from the lipoprotein binding to the sperm membrane via the charged protein moiety, which aids in stabilising the plasma membrane through the

Table 9

Recovery of spermatozoa and survival at 37°C after cold shock in CAPROGEN® containing 5% whole egg yolk or an equivalent amount of water-based lipoprotein extract (WTM) of egg yolk. Data from Vishwanath et al. (1992)

| Diluent                 | Recovery (%)     | Survival (h)     |
|-------------------------|------------------|------------------|
| CAPROGEN® (no egg yolk) | < 5 <sup>a</sup> | 2.5 <sup>a</sup> |
| CAPROGEN® (5% egg yolk) | 80               | 104 <sup>b</sup> |
| Water extract (WTM)     | 80               | 116 <sup>c</sup> |

Values with different superscripts differ,  $p < 0.05$ .

critical temperature zones, with the lipid portion acting as thermal insulation (Vishwanath et al., 1992). This water-based fraction was significantly better than egg yolk in maintaining survival of spermatozoa recovered after cold shock (Table 9; Vishwanath et al., 1992). Field fertility with the same fraction in ambient temperature stored semen where it was used as a thermal protectant also revealed a significant advantage (Table 10).

Evidence suggests that the whole lipoprotein molecule is required to protect spermatozoa during cold shock. Selective digestion of either the protein or the lipid fractions did not offer the same protection against cold shock (Watson, 1981; Prendergast et al., 1994). It is possible that the protein or the lipid fractions are unable to bind to the membrane and offer thermal protection on their own.

The amount of egg yolk commonly used in freezing media is between 15% and 30% on a volume to volume basis. This range in egg yolk concentration is based on results obtained by Van Demark et al. (1957) where the percentage of motile spermatozoa after freezing and thawing was similar for 15% and 30%, but was significantly lower at egg yolk concentrations above and below this range.

### 3.2.2. Ions

The ions in the storage medium are not mainly for ionic strength, but more for maintaining osmolarity (Watson, 1979). This purpose is adequately served by zwitterionic buffers, amino acids,  $\alpha$ -keto acids and a combination of salts and carbohydrates (Miller and Van Demark, 1954).

### 3.2.3. Cryoprotectants

**3.2.3.1. Glycerol.** The discovery of the protective action of glycerol (Polge et al., 1949) was an important technological breakthrough, as it reduced the mechanical damage to

Table 10

Fertility of semen from five sires diluted in CAPROGEN® containing 5% egg yolk or an equivalent amount of water-based egg yolk extract (WTM). Data from Vishwanath et al. (1992). Ejaculates were split between the two treatments and fertility measured as % NRR at 24 days

| Diluent                 | No. of inseminations | % NRR             |
|-------------------------|----------------------|-------------------|
| CAPROGEN® (5% egg yolk) | 8849                 | 66.6              |
| Water extract (WTM)     | 6195                 | 68.5 <sup>a</sup> |

<sup>a</sup> Indicates significantly different to CAPROGEN® ( $p < 0.05$ ).

spermatozoa during the freezing process. Glycerol and other penetrating cryoprotectants such as DMSO reduce cell damage by preventing the concentration effects of extracellular media. Colloids such as sucrose and dextran on the other hand, are non-penetrating cryoprotectants inducing the formation of ice crystal lattices, which are thought to form external shields around sperm membranes (Karow and Webb, 1965; Schmehl et al., 1986; Nicolajsen and Hvidt, 1994). The role of cryoprotectants is a matter of substantial debate and the current thinking on the nature of the protective effects of penetrating and non-penetrating cryoprotectants are discussed by Watson (1995).

Glycerol is the most widely used cryopreservative for bull spermatozoa. Conventional methods of freezing in 0.25- or 0.5-ml straws have used approximately 7% glycerol as the optimum concentration for citrate-yolk and Tris-egg yolk extenders (Pickett and Berndtson, 1974, 1978; Rodriguez et al., 1975; Watson, 1979, 1990; De Leeuw et al., 1993). The optimum concentration of glycerol for freezing bovine semen is also influenced by other components in the diluent (Woelders et al., 1997). Suffice to say, glycerol concentrations for freezing have been between 0.25 M (2.25%) and 1 M (9%) with many studies demonstrating toxicity beyond this concentration (Fahy, 1986).

**3.2.3.2. Sugars and polyols.** Nagase et al. (1964) found that xylose, fructose, glucose, galactose, maltose, sucrose and raffinose were all effective for rapid freezing of bull semen. Sugars perform several functions in the diluent. They add osmotic pressure to the medium and act as cryoprotectants (Watson, 1979). Polyols, like glycerol and several sugars, form hydrogen bonds with the polar head groups of membrane lipids (Crowe and Crowe, 1984; Crowe et al., 1985). The main effect of sugars and polyols is their ability to replace the water molecule in the normally hydrated polar groups and this property helps to stabilise the membrane during transition through the critical temperature zones (Woelders, 1997). Sugars, like glycerol, alter the mechanical properties of the diluent by increasing its viscosity. This prevents the eutectic crystallisation of solutes and increases the glass-forming tendency of the medium, a property used increasingly in vitrification media (Nicolajsen and Hvidt, 1994). A recent study (Woelders et al., 1997), using trehalose and sucrose, demonstrated a significant interaction between cooling rates and the presence of sugars. In this study, an isotonic sugar medium where the Tris-citrate components were substituted with sucrose and trehalose was significantly superior to a Tris-citrate-egg yolk medium in preserving acrosome integrity. The suggestion was that by using a cooling rate in the optimum range of 76–140°C/min a further improvement in viability after thawing could be achieved. Trehalose is a common substitute for water molecules in the dehydration process and is the sugar found in many organisms that survive anhydrobiosis (Woelders et al., 1997).

#### **3.2.4. Milk diluents**

Milk proved to be a suitable diluent for storage of bull semen both in the liquid and frozen state (Salisbury and Van Demark, 1961; Melrose, 1962). A 10% whole milk or skim milk diluent in combination with 7% glycerol and antibiotics has been very successfully used as a diluent for freezing semen for many years. Along with the Tris-egg yolk-citrate diluent developed by Davis et al. (1963a), milk diluents have been routinely used in many AI centres to freeze semen (Almquist et al., 1954; Ahmad and

Foote, 1985). Various studies have attempted to improve the whole milk or skim milk extenders, but have generally been unsuccessful (Foote et al., 1993). The main disadvantage with milk extenders is the poor visibility of spermatozoa under a microscope, which makes post-thaw evaluations difficult. It is for this reason that egg yolk diluents in combination with Tris and citrate are preferred.

### *3.3. Freezing protocols: processing, cooling rates, freezing and thawing*

#### *3.3.1. Initial processing and temperature equilibration*

Methods of processing of semen immediately after collection vary considerably. It is mostly guided by the methods of use that have suited the organisation, requirements for disease control and semen export protocols. Based on these requirements, procedures vary between organisations that are actively engaged in the international trade of bovine semen. Semen is usually diluted at 30°C to 37°C with an equilibrating medium containing all the basic constituents of the freezing diluent. In most cases, glycerol is omitted from this medium. The ratio of dilution is approximately 1:5 semen/diluent. This procedure is necessary to provide a buffering medium for spermatozoa, some antibiotic protection and to provide thermal insulation during the subsequent cooling. The Certified Semen Services (CSS, USA) procedure for entry of all imported semen into the USA requires undiluted semen to be treated with antibiotics before initial dilution (Lorton et al., 1988). It is generally considered that the slow cooling of diluted semen to 5°C before freezing has beneficial effects (Ennen et al., 1976; Gilbert and Almquist, 1978). This period, during which glycerol is usually added is sometimes erroneously referred to as the glycerol equilibration period. Glycerol equilibrates quite rapidly across the cell membrane at 5°C and can be added at any time during the cooling period (Martin, 1965; De Leeuw et al., 1993). Once the diluted semen attains the holding temperature of 5°C, the mixture is extended to the final sperm concentration with the same medium containing glycerol. All subsequent manipulations such as filling and sealing into straws are usually conducted at 5°C. The final sperm concentration per milliliter of diluted semen differs between organisations. It also depends on stipulations by importing countries. The relationship between the total number of spermatozoa per breeding unit and threshold fertility is discussed in a later section. The average sperm concentration is between 10 and 20 million per breeding unit, depending on the demand of semen from individual sires.

#### *3.3.2. Effect of cooling rates*

A two factor theory accounts for the effects of cooling rates on spermatozoa (Mazur, 1965; Mazur et al., 1972). A slow cooling rate exposes spermatozoa to the 'solution effects' such as increasing salt concentration, increasing osmolality and changing pH. Fast cooling may not allow intracellular water to pass out, leading to intracellular ice nucleation (Mazur, 1963, 1977; Mazur et al., 1972). In practice, the cooling rate at which freezing damage occurs in spermatozoa is several orders lower than the calculated rate at which ice nucleation should occur. A recent report (Woelders, 1997) shows a clear relationship between survival of bull spermatozoa and cooling rate (Fig. 4). The

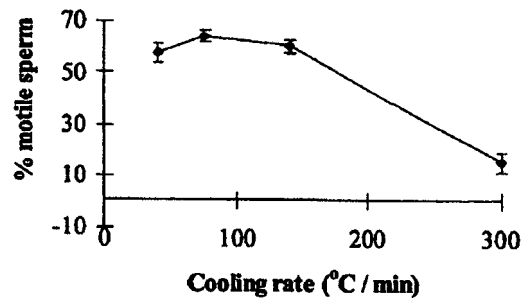


Fig. 4. Relation between % motile bull spermatozoa post-thaw and the cooling rate. Values are means  $\pm$  se for five bulls. Data from Woelders (1997).

optimum cooling rate was 100°C/min, and this is greater than the optimum of 26–52°C/min suggested earlier for bovine spermatozoa (Robbins et al., 1976). A further detailed study by Woelders et al. (1997) suggests a significant interaction between the osmolality of the medium and cooling rate. Supra-optimum cooling rates are tolerated at higher osmolalities where the medium contains sucrose or trehalose.

### 3.3.3. Methods of freezing

The technique of freezing semen in 0.25- or 0.5-ml straws in liquid N<sub>2</sub> is now universal (Cassou, 1964). This technology is well established and all the equipment required for filling semen in straws and racking them up for freezing is readily available. The appliances used for freezing of semen range from static freezers to controlled programmable freezers. Although there has been considerable research in determining optimum cooling rates and adapting cooling curves for individual bulls, there has been very little progress in the practical sense. The programmable freezers currently available cannot reliably alter or allow customisation of cooling rates for a large batch of straws in a production environment (Wagtendonk, personal communication). Many semen processing organisations continue to use static vapour freezers where the straws containing semen are subjected to uncontrolled freezing conditions, depending on the distance of the straw chamber from the liquid N<sub>2</sub> layer. In these freezers, the rate of decrease in temperature is between 150°C/min and 300°C/min. The advantage of the static vapour freezer is that all the straws in any given freezing cycle are subjected to the same cooling rates, because the straws are placed in one layer above the liquid N<sub>2</sub> level. In programmable freezers, the straws are placed in more than one layer, and this contributes to considerable variation in cooling rates in individual layers within a freezing cycle (Wagtendonk, personal communication).

### 3.4. Thawing of semen

The general theory is that rapid thawing of semen is advantageous to prevent injury during rewarming. Mazur (1984) observed that rapid thawing prevents the possibility of re-crystallisation of water molecules, which could be injurious to cell membranes. A

Table 11

Fertility of frozen semen from nine bulls thawed at 35°C or at ambient temperature. Data from Vishwanath (1998) and fertility expressed as % NRR at 24 days

| Temperature of thawing | Number of inseminations | % NRR      |
|------------------------|-------------------------|------------|
| Ambient (14 ± 3°C)     | 1182                    | 72.7 ± 1.3 |
| 35 ± 1°C               | 1105                    | 71.4 ± 1.4 |

potential problem in slow thawing is the osmotic change due to ingress of water during the process, as this is considered to be more damaging than water egress during freezing (Curry and Watson, 1994). Studies by Holt and North (1994) have also confirmed this observation with ram spermatozoa. There are some practical considerations regarding the temperature at which semen used in field conditions should be thawed to obtain maximum fertility. This is a question often asked with many anecdotal references to higher fertility with semen thawed at higher temperatures (> 25°C). There are some earlier studies evaluating fertility of semen thawed at temperatures of 30°C, 15°C and 4°C (Arnott, 1961). The 60–90 day NRR followed the trend of 4°C > 15°C > 30°C in thawing temperature. Other studies have also suggested a lower temperature of around 4°C as favourable for thawing, compared to higher temperatures of around 15°C (Pickett et al., 1965; Pickett and Berndtson, 1978). A recent field trial to determine the effect of thawing semen at 35°C compared with ambient temperature (14 ± 3°C) showed no difference in NRR between the two thawing temperatures (Table 11; Vishwanath, 1998).

### 3.5. Re-dilution of bulk frozen semen

The concept of re-dilution of frozen-thawed semen was first explored by James and Fyvie (1955). The idea was to develop it as a convenient method to harness the out-of-season production potential of a bull. The initial trials were not very successful and the fertilising ability of re-diluted semen could be retained for only a few hours. The technique was later successfully modified for field use (Shannon, 1972; Macmillan et al., 1978). In this system, the semen was frozen in straws at high sperm concentrations in an egg yolk–citrate–glycerol diluent and re-diluted with CAPROGEN® upon thawing (Shannon, 1965) and before using as liquid semen. This technology was used very efficiently for a number of years in many isolated areas in New Zealand. The AI technician diluted the contents of a 0.25-ml straw in a test tube containing 5 ml of CAPROGEN® and completed the day's inseminations. Inseminations of 0.5 ml volumes were made by using a syringe pipette. At the end of the day, the remaining diluted semen was discarded, as earlier trials had shown a significant decrease in fertility of the re-diluted semen stored overnight (Shannon, 1978). The process has been improved so that large volumes of concentrated semen (5–25 ml) can be bulk frozen (patent pending) and re-diluted in the production laboratory on the days required for use. This is then dispatched as standard liquid semen and used within 60 h of re-dilution. The sperm concentration of the re-diluted material range between 6 and 10 million spermatozoa/in-

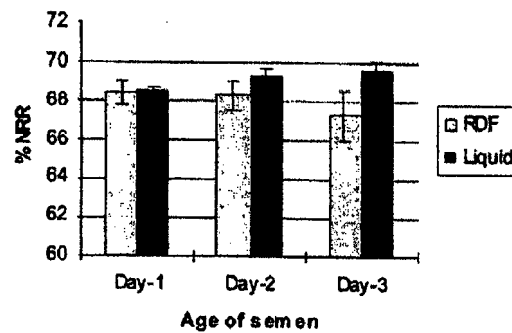


Fig. 5. NRRs (24 day) for re-diluted frozen (RDF) semen and liquid semen on days 1–3 after thawing/re-dilution and collection, respectively. Data from Vishwanath et al. (1996).

seminate. The results with re-diluted frozen semen are shown in Fig. 5. The percentage NRR for the first 2 days of use was not significantly different for the two types of semen, but was significantly lower on day 3 of use with re-diluted semen ( $p < 0.05$ ). Two aspects that could potentially affect this system are the significant bull  $\times$  sperm concentration interaction, which determines the final sperm concentration in the inseminate and, secondly, the bull  $\times$  age of semen interaction, which limits the number of days semen can be used in a diluted state in the field.

### 3.6. Effect of sperm numbers on fertility of semen stored in the liquid or frozen state

Bulls differ in their inherent fertility and follow similar fertility trends whether their semen is diluted and stored in liquid or frozen state. This effect is shown in Fig. 6. The percentage NRR of liquid and frozen semen for the same 11 bulls at optimum sperm concentration (liquid 2.5 million/inseminate, frozen 20 million/inseminate) range between 62% and 72% (Shannon and Vishwanath, 1995). When sperm concentrations were decreased to sub-optimum levels (liquid 0.5 million/inseminate, frozen 5 million/inseminate), fertility declined by an average of 7% for liquid semen and by 7.9% for frozen semen. There was a significant bull  $\times$  dose rate interaction in the frozen semen system, which was not seen, in the liquid system ( $p < 0.01$ ). This clearly highlights the fact that semen ejaculates from different bulls inherently differ in their susceptibility to freezing as measured by their NRR. Further evidence suggests that this could be due to a reduced probability of fertilisation or an altered pattern of survival of frozen-thawed spermatozoa in the female reproductive tract (Shannon and Vishwanath, 1995). A similar bull  $\times$  dose rate interaction for frozen semen was observed by den Daas (1992). Much greater differences between bulls regarding NRR was seen at lower than at higher sperm concentrations.

The true relationship between the number of spermatozoa inseminated and fertility was first proposed by Salisbury and Van Demark (1961). This was further explained by a model proposed by Schwartz et al. (1981), which relates the number of spermatozoa to the probability of conception, based on a Poisson distribution. The validity of the model

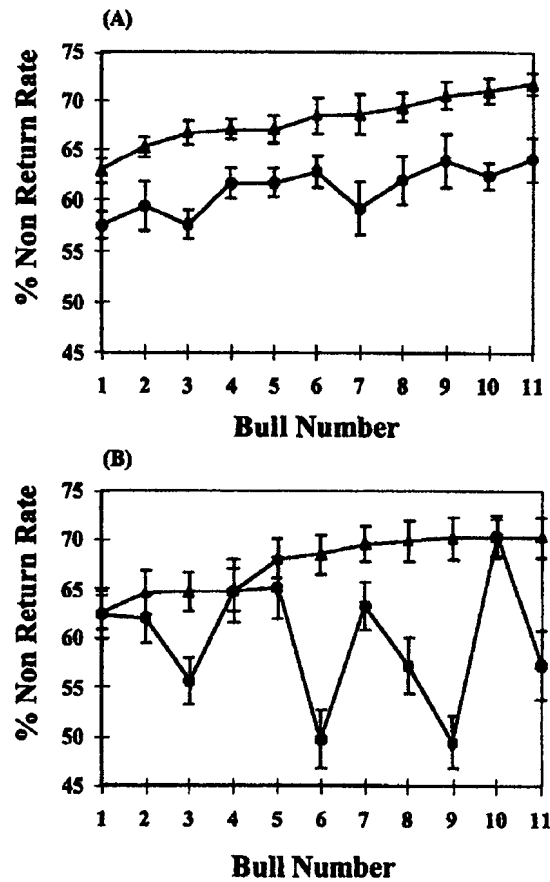


Fig. 6. Variation in NRR between bulls at optimum (▲) and suboptimum (●) sperm concentrations in the liquid (A) and frozen (B) treatments. (Data from Shannon and Vishwanath, 1995.)

was reaffirmed by the results of a series of field trials (den Daas, 1992). Bulls differ in their maximum NRR value, and this is reached as the number of spermatozoa per inseminates increases. The rate at which this maximum value is approached also differs between bulls. The added variation to this is the inherent fertility difference between sires that occur depending on time of insemination. There is a significant interaction between insemination time, sperm concentration, stage of oestrus of the cow (pre- or post-oestrus) and fertility of individual sires (Macmillan and Curnow, 1977).

The differences in sire fertility have been illustrated in two separate studies by Macmillan and Watson (1975), and Macmillan and Curnow (1977). The stage of oestrus at insemination did not significantly influence the results for sires of above average fertility, but below average sires had reduced conception rates with mid and early oestrus inseminations. The observation suggests that there could be an *in vivo* decline in fertilising ability of spermatozoa, which differs between sires.

The reasons for the differences in the response to dilution rate are not known. It is possible that individual bulls react differently to a single freezing protocol that is normally used by semen processing companies. Perhaps, customising freezing protocols for individual bulls could help in reducing the dose rates of frozen semen (Parkinson and Whitfield, 1987; den Daas, 1992). The fact that higher numbers of spermatozoa are required for insemination with frozen semen due to losses in the freezing process is not unexpected. The number of live spermatozoa required for insemination is far lower when using liquid than frozen semen; at the same dose rate of 2 million motile sperm, NRRs for liquid and frozen semen were 68.1% and 59.7%, respectively.

3.7. Advantages and disadvantages of liquid stored and frozen–thawed semen

The relative advantages and disadvantages in the field usage of liquid stored and frozen–thawed semen are listed in Table 12. The most obvious advantage of liquid stored semen is one of sperm numbers, and this has been the main impetus in further refining liquid semen technology for extensive use in New Zealand and other countries. An average ejaculate of 5 ml containing 1.5 billion spermatozoa/ml will yield between 350 and 500 frozen semen straws at a dose rate of 15 to 20 million spermatozoa/inseminate. On the other hand, the same ejaculate as liquid semen will yield 7500 straws at a dose rate of 1 million/inseminate and 3750 straws at the higher sperm concentration. The storage costs of liquid semen are very low. If semen utilisation is high within the shelf-life period after dilution, the economic advantages are quite significant (Shannon, 1978; Curson et al., 1991). The obvious disadvantage with liquid stored semen is the limited shelf-life. By contrast, the most important advantage of frozen–thawed semen is the possibility of long-term storage capability. This allows for extensive testing of the processed semen, and is a reliable method for genetic insurance of valuable bulls. The biggest disadvantage in a high demand situation is the high sperm numbers required for insemination to maintain the same level of fertility as for liquid stored semen. In New Zealand, a unique system of seasonal breeding operates. More than 2.5 million inseminations are performed in a 16- to 20-week period during the Spring months of September to Summer (Fig. 7). More than 85% of these inseminations

Table 12  
Relative advantages and disadvantages of liquid-stored and frozen–thawed semen

| Liquid-stored semen       | Frozen–thawed semen |
|---------------------------|---------------------|
| <i>Advantages</i>         |                     |
| Low sperm numbers.        | Long term storage.  |
| High sire utilisation.    | Flexibility of use. |
| Inexpensive storage.      |                     |
| Ease of use in the field. |                     |
| <i>Disadvantages</i>      |                     |
| Limited shelf life.       | High sperm numbers. |
|                           | Expensive to store. |

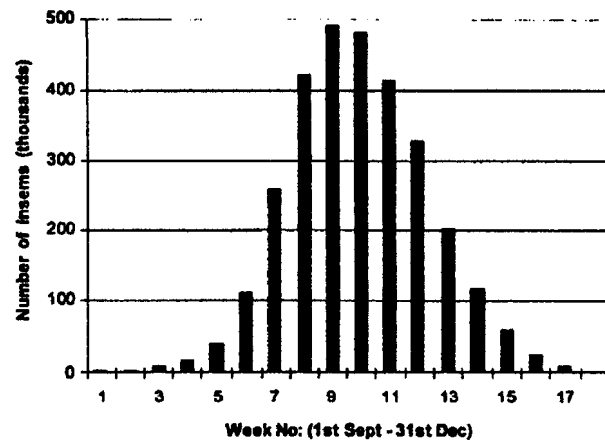


Fig. 7. The distribution of inseminations in New Zealand during the spring mating period (1st September–31st December). Data from Vishwanath et al. (1996).

are with liquid stored semen, which has a 4-day shelf-life (Curson et al., 1991; Vishwanath et al., 1996).

### 3.8. Semen packaging

The current methods for semen packaging are largely based on the French mini-straw, the 0.25-ml paillette (Cassou, 1964). The procedure has been in operation for many years and has generally worked well for packaging frozen and liquid semen in many countries (Chupin and Schuh, 1993). The straw allows essential details of the sire to be recorded and this has become mandatory for semen traded internationally. The packaging method for liquid semen in New Zealand is the minitub straw sealed at both ends with glass beads (Minitüb, Germany).

Freezing of semen in pellets was first described by Japanese workers (Nagase and Niwa, 1964; Nagase et al., 1964) and is still used in some countries for storing semen from young sires and for local use. This procedure is not widely practised because details of the sire cannot be easily recorded on a pellet.

## 4. Commercially available diluents

Most AI companies prepare their own semen diluents with minor modifications to suit their requirements. In the last few years, a few proprietary brands have been available and they are listed below. The move to eliminate egg yolk from the system has been quite strong because of a perceived health risk associated with a biological material being included in the diluting media. However, suitable alternatives have not been as successful as either milk or egg yolk (van Wagendonk-de Leeuw et al., 2000). Some of the diluents listed below are dual purpose and recommended for both liquid and frozen storage of semen. The list is by no means exhaustive; nor has a detailed market survey

been conducted on the relative merits of all the media available. The common ones cited in literature are the following.

(1) Biladyl — supplied by Minitüb Germany and used with frozen semen. It consists of two fractions A and B for step-wise dilution of semen with addition of egg yolk, water and antibiotics as prescribed by CSS.

(2) Triladyl — supplied by Minitüb Germany and used with frozen semen. This is similar to Biladyl and is used as a one-step dilution medium.

(3) Biociphos — supplied by IMV, L'Aigle, France. It is a one-step dilution medium containing soy bean extract, replacing the egg yolk fraction.

(4) Laciphos — supplied by IMV. It is a skim milk-based powder medium requiring addition of water. Both Biociphos and Laciphos are supplied with antibiotic fractions that satisfy either the USA (CSS-certified antibiotic) or the EEC antibiotic formulae.

(5) Tris concentrate — Gibco BRL, Manufactured by Holland Genetics, The Netherlands. This is a 5 × concentrate of the popular Tris-based medium (Davis et al., 1963a) and modified by De Leeuw et al. (1993). It is the most common diluent for freezing semen. The Tris diluent has been used also for liquid semen (Foote, 1978).

(6) CAPROGEN® concentrate — concentrated liquid semen diluent for storage at ambient temperatures (18°C to 24°C) supplied by Livestock Improvement, New Zealand. It is a diluent extensively used for liquid storage of semen with no drop in fertility over a 4-day period (Curson et al., 1991).

## 5. Future directions

### 5.1. Liquid stored semen

Major gains can be made with liquid semen technology if the decline in fertility upon storage at ambient temperature is halted or reduced. To this end, the physiological processes that contribute to aging of spermatozoa upon storage *in vitro* need to be understood. Reactive oxygen species, which are more likely to be generated in an ambient temperature system have to be contained in the media in which the spermatozoa are suspended. Intracellular activity could also contribute to the production of these free radicals and the slowing down of respiratory activity could be beneficial.

### 5.2. Frozen semen

The future direction of research on freezing semen should be directed towards improvement of freezing protocols to allow a lower number of spermatozoa to be included per breeding unit. The current freezing protocols require from 40 to 100 million spermatozoa/ml of diluting medium and each inseminate contains between 10 and 25 million spermatozoa. For any substantial gain to be made in this direction, future work needs to concentrate on diluent composition and repeatable cooling rates to protect the sperm cells during the freezing process.

### 5.3. Alternative storage technologies

Storage of semen in frozen state is expensive, as liquid N<sub>2</sub> and well-insulated containers are essential. The on-going cost of storing semen in liquid N<sub>2</sub> can be

prohibitive. There has always been an interest in developing alternative strategies for long-term storage of bovine spermatozoa, and experiments on desiccation, vitrification and freeze drying have been attempted in the past with limited success (Larson and Graham, 1976; Jeyendran et al., 1981, 1983). A recent review by Holt (1997) discusses the future requirements for preservation of germplasm and how these strategies need to be re-visited in the light of modern knowledge on the response of sperm membranes to severe manipulations. The next breakthrough in semen storage technology will almost certainly involve some of these strategies.

## 6. Concluding remarks

The use of liquid stored semen allows high utilisation of individual sires. This is possible because of the low sperm numbers required to maintain fertility and the extended shelf-life of up to 4 days of use in the field. If the effects of semen age can be overcome, the efficiency and utilisation of liquid stored semen could be increased quite significantly. The essential difference between liquid and frozen-thawed semen lies in the response of bulls to sub-optimum dose rates. While all bulls respond similarly to sub-optimum dose rates with liquid stored semen, the response varies greatly when frozen-thawed semen is used. This means that higher than required dose rates are recommended for frozen-thawed semen because of the possibility of a bull  $\times$  dose rate interaction. The use of bulk-frozen semen re-diluted and used as liquid semen is an option to combine the convenience of frozen semen technology with the efficiency of liquid semen.

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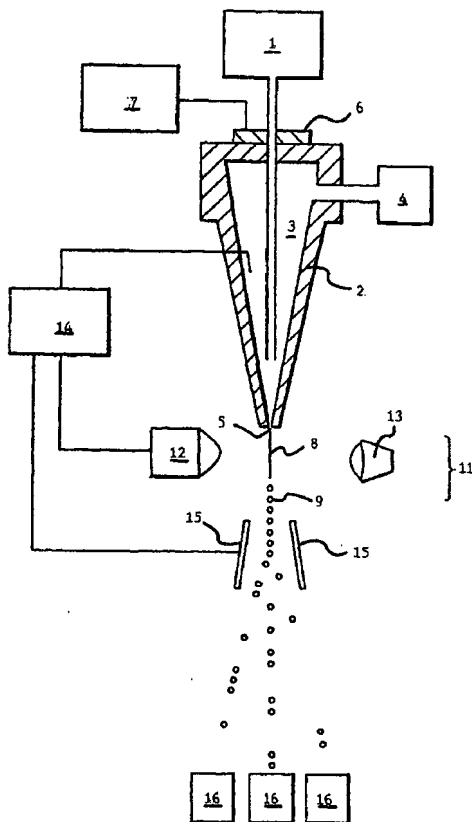
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(54) Title: LOW PRESSURE SPERM CELL SEPARATION SYSTEM



(57) Abstract: A sperm cell process system providing to generate sperm cell insemination samples having controlled sperm cell fertility characteristics of sperm cells.

WO 2004/012837 A2

## LOW PRESSURE SPERM CELL SEPARATION SYSTEM

This International Patent Cooperation Treaty Patent Application claims the benefit of United States Provisional Patent Application No. 60/400,971, filed August 1, 2002.

## 5 I. TECHNICAL FIELD

Sperm cell insemination samples having selectably controlled sperm cell fertility characteristics produced through entrainment in a fluid stream having correspondingly selectably adjustable flow characteristics and methods of assessing comparative of sperm  
10 cell insemination sample fertility.

## II. BACKGROUND

Pre-selection of sex has been accomplished in many species of livestock following the development of safe and reliable methods of separating sperm cells into enriched X chromosome bearing and Y chromosome bearing populations. See for example methods  
15 and apparatus disclosed by WO 00/06193; WO 02/043574; WO 01/85913; WO 99/33956; WO 01/40765; WO 98/34094; WO 99/42810; WO 02/043486.

A significant problem with sex selected sperm cells may be that separation of sperm cells at rates sufficient to produce sex selected insemination samples or sex selected inseminates which are viable or sufficiently fertile for commercial application by  
20 conventional technology has necessitated increasing fluid stream pressure of flow cytometers or flow sort instruments to about 50 pounds per square inch. With respect to sperm cells of many species of mammals entrained in fluid streams having flow characteristics resulting from this application of pressure the viability, motility, or other fertility characteristics altered.

25

Another significant problem with sex selected sperm cell inseminates or sex selected sperm cell insemination samples can be the vast difference in sperm cell fertility characteristics which can vary greatly between samples. As such, success of artificial insemination performed under substantially identical conditions can result in  
30 correspondingly different pregnancy rates.

Another significant problem with existing sperm cell sex selection technology can be the lack of an assay from which fertility of sex selected sperm cells can be compared directly in-vivo (for example, in conjunction with artificial insemination procedures) and in-vitro (for example, in conjunction with IVF procedures).

5 The instant invention addresses the variety of problems associated with reduced sperm cell fertility spermatozoa that have been separated into enriched X-chromosome bearing and Y-chromosome bearing populations and the lack of heterospermatic assays to compare function and fertility of separated or sorted sperm cells, and in particular flow sorted sperm cells.

10

### III. DISCLOSURE OF THE INVENTION

Accordingly, the broad object of the invention provides devices and methods of using such devices to control sperm cell fertility characteristics of sperm cells isolated from semen obtained from a male of species of mammal, such as motility, viability, fertilization rate, cleavage rate, blastocyst rate, or the like.

Providing controlled sperm cell fertility in accordance with the invention can be achieved with the sperm cells obtained from numerous and varied species of mammals, including without limitation, mammals selected from the group consisting of a bovine species of mammal, an equine species of mammal, an ovine species of mammal, a canine species of mammal, a feline species of mammal, a swine species of mammal, a marine species of mammal, a deer species of mammal, a primate species of mammal, a goat species of mammal, or a species of mammal listed by Wilson, D. E. and Reeder, D.M., Mammal Species of the World, Smithsonian Institution Press, (1993), hereby incorporated by reference herein.

With respect to certain embodiments of the invention, controlled sperm cell fertility characteristics comprises affirmative selection of fertility characteristics in advance of isolating sperm cells from the semen of the male of the species of mammal and application of the invention to alter sperm cell fertility characteristics to provide the fertility characteristics desired. With respect to other embodiments of the invention,

sperm cell treatment conditions are selected within a broader range of sperm cell treatment conditions that can be used treat sperm cells of a particular species of mammal to obtain sperm cells having controlled fertility characteristics. Controlled fertility characteristics can comprise a desired proportion of motile sperm cells, intact acrosomes, viable sperm cells within a population of treated sperm cells; or can comprise a desired cleavage rate of oocytes or rate of blastocyst formation when treated sperm cells are utilized to fertilize oocytes in vitro; or can comprise a desired pregnancy rate or sex ratio of offspring when treated sperm cells are utilized for artificial insemination. With respect to certain embodiments of the invention, a greater proportion of motile sperm cells, a greater proportion of viable sperm cells, a greater proportion of intact acrosomes, or a greater number of fertile sperm cells within a treated sperm cell population can be achieved compared to conventional treatment of the same of sperm cell population. Certain embodiments of the invention allow provision of sperm cells having controlled fertility characteristics which are not substantially different than, or are substantially comparable to, the fertility characteristics of sperm cells in fresh ejaculated semen. In other instances, application of certain embodiments of the invention can if desired result in sperm cells having controlled fertility characteristics which are substantially different than those of sperm cells of fresh ejaculated semen. In particular, certain embodiments of the invention can be used to provide bovine sperm cells having controlled fertility characteristics or can be used to provide equine sperm cells having controlled fertility characteristics, which if desired can be provided with fertility characteristics substantially comparable to the fertility characteristics of bovine sperm cells or equine sperm cells within freshly ejaculated bovine or equine semen.

Another broad object of the invention can be to provide sperm cell insemination samples having controlled sperm cell fertility characteristics, such sperm cell insemination samples, without limitation, can be configured for artificial insemination of a female of a species of mammal, in vitro fertilization of oocytes, or intracytoplasmic injection of sperm cells, or the like.

30

Another broad object of the invention can be to provide methods of sex selecting sperm cells that can provide affirmative control of sperm cell fertility characteristics such as motility, viability, fertilization rate, cleavage rate, blastocyst rate, or the like. One aspect of this broad object of the invention can be to provide flow cytometry or cell

sorting devices or methods of flow cytometry or cell sorting which allows affirmative control of the fertility characteristics of sex selected sperm cells.

Another object of the invention can be to provide sex selected bovine sperm cell  
5 insemination samples having controlled sperm cell fertility characteristics configured for artificial insemination of a female of a bovine species of mammal containing between about 100,000 and about 3,000,000 sex selected bovine sperm cells having controlled sperm cell fertility characteristics.

10 Another object of the invention can be to provide sex selected equine sperm cell insemination samples having controlled sperm cell fertility characteristics configured for artificial insemination of a female of an equine species of mammal containing between about 5,000,000 and about 50,000,000 sex selected bovine sperm cells having controlled sperm cell fertility characteristics.

15 Another significant object of the invention can be to provide devices or methods of maintaining controlled sperm cell fertility characteristics of sperm cells with respect to processing of sperm cells, storage of sperm cells, or use of sperm cells, including, but not limited to, insemination of female mammal(s) or fertilization of oocyte(s).

20 Another significant object of the invention can be to provide methods of artificially inseminating females of a species of mammal with sperm cell insemination samples having controlled sperm cell fertility characteristics. With respect to certain embodiments of the invention, methods of insemination with a low or reduced number of sperm cells  
25 having controlled fertility characteristics compared to the usual number or typical number of sperm cells used in such artificial insemination procedures whether or not such sperm cells are separated into enriched X chromosome bearing or Y chromosome bearing sperm cell populations.

30 Another broad object of the invention can be to provide a method of assessing comparative fertility of sperm cell populations. Certain embodiments of the invention provide a method of assessing comparative fertility of sperm cells from different males of a species of mammal when sperm cells from each male are exposed to substantially the same flow cytometric treatment. Other embodiments of the invention provide a method of

assessing comparative fertility of sperm cells from the same male of a species of mammal which are exposed to different flow cytometric treatments. Certain embodiments of the invention provide methods of showing comparative fertility of sperm cells having controlled fertility characteristics.

5

Naturally, further significant objects of the invention are made clear in the proceeding description of the invention.

#### IV. BRIEF DESCRIPTION OF DRAWINGS

10 Figure 1 is a schematic diagram of a sorter system according to a flow cytometer separation technique for the present invention.

Figure 2 is a diagram of the entrained cells in the free fall area of a typical flow cytometer.

15

#### V. MODE(S) FOR CARRYING OUT THE INVENTION

A semen or sperm cell process system to maintain, enhance, assay, test, or determine the biological, chemical, physical, physiological, or functional attributes of sperm cells within the context of various collecting, handling, storage, transportation,  
20 separation, or insemination procedures.

An embodiment of the invention can comprise obtaining a sperm cells from a species of mammal as broadly defined above. The sperm cells can then be entrained in a fluid stream having flow characteristics. The fluid stream within a conduit has flow characteristics influenced by the rheological properties of the fluid stream, the  
25 configuration or geometries of the conduit in which the fluid stream flows, as well as external forces applied to the fluid stream such as hydrostatic pressure, oscillatory vibrations, piezoelectric vibrations, oscillations in heat, or the like.

Importantly, these flow characteristics of the fluid stream contribute to the amount  
30 of pressure required to move fluid within the conduit. As a non-limiting example, in flow cytometry fluid moves within a relatively large cross sectional area and then within a relatively small cross sectional area past an analysis interface to a final collection point.

This type of configuration or geometry along with rheologic properties of the fluid stream can create localized forces such as compressive forces, sheer forces, or the like, which can have influence the physical integrity of particles such as sperm cells entrained  
5 in the fluid stream.

With respect to those embodiment of the invention which include components of or steps involving flow cytometry or cell sorting as a means to analyze, separate based upon a sperm cell characteristic, sex select, or other wise process sperm cells, a conceptual non-  
10 limiting flow cytometer or cell sort instrument is shown by Figure 1.

A flow cytometer or cell sort instrument includes all or a portion of the components shown by Figure 1 including with out limitation, a sperm cell source (1) which acts to establish or supply sperm cells to analyze, separate, control fertility  
15 characteristics, or be otherwise treated.

Sperm cells are deposited within a nozzle (2) in a manner such that sperm cells are surrounded by a sheath fluid (3). Any sheath fluid compatible with the flow cytometer or flow sort instrument and which provides an acceptable environment for sperm cells during  
20 flow analysis or processing can be utilized with the invention, including without limitation, sheath fluids which contain, individually or in various combinations, a phosphate buffered saline, a citrate solution (such as a 2.9% sodium citrate solution), or a HEPES buffered solution.

25 The sheath fluid (3) is usually supplied by some sheath fluid source (4) so that as the sperm cell source (1) supplies sperm cells, the sheath fluid (3) is concurrently fed through the nozzle (2). In this manner, the sheath fluid (3) forms a sheath fluid environment for the cells. Since the various fluids are provided to the flow cytometer at some pressure, they flow out of nozzle (2) and at the nozzle orifice (5).

30

By providing some type of oscillator (6) which may be very precisely controlled through an oscillator control (7), pressure waves may be established within the nozzle (2) and transmitted to the sheath fluid exiting the nozzle (2) at nozzle orifice (5). Since the oscillator (6) thus acts upon the sheath fluid (3), the stream (8) exiting the nozzle orifice

(5) eventually and regularly forms drops (9). Because the cells are surrounded by a sheath fluid environment, the drops (9) may contain within them individually isolated sperm cells (10).

5        Since the droplets (9) generally contain isolated sperm cells (10), the flow cytometer or cell sorter instrument can distinguish between and separate droplets based upon a distinguishing sperm cell characteristic(s) of the sperm cell contained within a droplet (9). This is accomplished through a sperm cell sensing system (11). The sperm cell sensing system involves at least some type of detector (12) which responds to sperm  
10       cells contained within each droplet (9).

One type of sperm cell sensing system (11) is as discussed at length in United States Patent No. 5,135,759 to Johnson, hereby incorporated by reference herein. As the Johnson patent explains for sperm cells, the cell sensing system (11) may cause an action  
15       depending upon the relative presence or relative absence of a particular dye which may be excited by some stimulant such as the beam of a laser (13). While each type of sperm cell can be stained with a dye, the differing length of the X-chromosome and the Y-chromosome causes different levels of staining. Thus, by sensing the degree of dye present in each sperm cells it is possible to discriminate between X-chromosome bearing sperm  
20       and Y-chromosome bearing sperm by their differing emission levels. Alternate optics, detection and sperm cell analysis systems are known which can also be used in accordance with the invention and it is intended that the description provided by the Johnson patent is for illustrative purposes so that the numerous and varied uses of the invention can be understood. See also, WO 01/85913 , hereby incorporated by reference herein.

25

In order to achieve the ultimate separation and isolation of the appropriate cells in a flow cytometer or cell sort instrument separation technique, the signals received by sensor (12) are fed to some type of sorter discrimination system (14) which very rapidly makes the decision and can differentially charge each droplet (9) based upon whether the  
30       desired cell does or does not exist within that droplet (9). In this manner the sorter discrimination system (14) acts to permit the electrostatic deflection plates (15) to deflect droplets (9) based on whether or not they contain the a sperm cell having certain sperm cell characteristics. As a result, the flow cytometer or cell sorter instrument acts to separate the cells by causing them to land in one or more collection containers (16). Thus

by sensing some property of the sperm cells the flow cytometer or cell sorter instrument can discriminate between cells based on a particular characteristic and place them in the appropriate collection container (16). In certain flow cytometers or cell sorter instruments, the X-bearing sperm droplets are charged positively and thus deflect in one direction, the  
5 Y-bearing sperm droplets are charged negatively and thus deflect the other way, and the wasted stream (that is unsortable cells) is uncharged and thus is collected in an undeflected stream into a suction tube or the like.

Now referring primarily to Figure 2, the process can be even further understood.  
10 As shown in that figure, the nozzle (2) emits a stream (8) which because of the oscillator (7) (not shown in Figure 2) forms drops (9). Since the cell source (1) (not shown in Figure 2) may supply sperm cells (10) which as described by Johnson can be stained (or in certain embodiments of the invention unstained as when using DIC technology), the light emission generated by the beam generated by laser (or illumination source when using  
15 DIC technology) (13) incident upon the dye (the sperm head when DIC technology is utilized) is differentially determined by sensor (12) so that the existence or nonexistence of a charge on each drop (9) as it separates from stream (8) can be controlled by the flow cytometer. This control results in positively charged, negatively charged, and uncharged drops (9) based upon their content. As shown in Figure 2, certain drops are shown as  
20 deflected drops (17). These deflected drops (17) are those containing sperm cells (10) which can be one or the other sex. They are then deposited in the appropriate collector (16) thereby generating a population of sex selected sperm cells.

Whether the fluid stream occurs within the context of a flow cytometer, cell sorter,  
25 or other device which entrains sperm cells within a fluid stream, the flow characteristics of the stream can be characterized and adjustment means for altering flow characteristics of the fluid stream can be introduced to increase or decrease forces such as compressive forces, sheer forces, or the like, such that particles entrained in the fluid stream can be physically, physiologically, functionally, or mechanically altered.

30

As such a selectably adjustable range of fluid stream characteristics for a flow path can be generated using the adjustment means and can be expressed as an incremental measure. For example, alteration of fluid stream characteristics within a flow cytometer or cell sort instrument context can be incrementally adjusted and measured in pounds per

square inch and typically allow the incremental increase or decrease in fluid stream pressure between about 20 pounds per square inch and 100 pounds per square inch with a commensurate increase or decrease in fluid stream or sheath fluid velocity.

5 In accordance with certain embodiments of the invention, sperm cells of a particular species of mammal are entrained in a fluid stream having adjustable fluid stream flow characteristics. Fluid stream flow characteristics are then selectably adjusted over an incrementally measured range in which the entrained sperm cells remain viable. Sperm cell fertility characteristics are then assessed for each of a plurality of sperm cell samples  
10 taken in correspondence to each of a plurality of flow characteristics generated within the measured range.

Subsequently, sperm cell fertility characteristics with respect to sperm cells from a species of mammal or individual members of a species of mammal can be controlled and  
15 sperm cell insemination samples can be generated having the desired sperm cell fertility characteristics.

For example, sperm cells from each of six bulls were stained with 125  $\mu$ m Hoechst 33342 for 45 min at 34°C, and bulk-sorted (passed through a flow cytometer or cell sorter  
20 instrument without sorting into subpopulations) or sorted with a flow cytometer having a nozzle with an internal diameter of 70  $\mu$ m into X-chromosome bearing or Y-chromosome bearing (or both) populations at about 95% accuracy with the fluid stream having a pressure of at 30 pound per square inch, 40 psi, or 50 psi. Lowering the fluid stream pressure from 50 psi to 30 psi reduced sorting rate by only 2 to 3%.

25 The sperm cells were subsequently cooled to 5°C and concentrated by centrifugation, loaded into 0.25 ml straws with about  $2 \times 10^6$  total sperm cells per 100  $\mu$ l column, and frozen using a vapor freezing method along with unsorted controls. The sperm cells in the straws were subsequently thawed.

30 Sperm cells were then evaluated with respect to various sperm cell fertility characteristics blindly by two observers at 30 and 120 min post-thaw for progressive motility, as well as by flow cytometry 105 min post-thaw, for percent live sperm cells by

PI stain, and by CASA analysis 120 min post-thaw using the Hamilton Thorne system. The entire procedure was twice replicated.

Factorial ANOVA indicated that both bull and pressure effects were significant  
5 (P<.005, Table I).

Table 1. Responses of sperm post-thaw to different system pressures during sorting.<sup>a</sup>

| Response                | Pressure (psi) |                  |      |                  |
|-------------------------|----------------|------------------|------|------------------|
|                         | 50             | 40               | 30   | Unsorted Control |
| 30 min motility (%)     | 44.7           | 48.6             | 49.6 | 52.1             |
| 120 min motility (%)    | 34.5           | 40.8             | 42.7 | 40.8             |
| 15 Live sperm (%)       | 51.7           | 55.7             | 57.8 | 58.5             |
| CASA total motility (%) | 25.1           | 37.2             | 40.9 | 34.8             |
| CASA ALH*               | 6.0            | 7.6 <sup>l</sup> | 7.8  | 8.8              |

20 <sup>a</sup> All statistically significant, P<.005.

\* Amplitude of lateral head displacement.

Higher numbers mean less stiff and more normal motility. There were typical differences among bulls in all responses. However, the bull by treatment interactions were small with  
25 one exception, meaning findings apply similarly to most bulls in the population. The flow characteristics of the fluid stream adjusted incrementally to increase pressure affected substantially all sperm cell fertility characteristics measured, however, only highly statistically significant ones are in Table 1.

30 As can be understood, there was significant change in sperm cell fertility characteristics between sperm cell samples taken at about 50 psi and at about 40 psi, and then a much smaller change between about 40 psi and about 30 psi, indicating that the effect on sperm cell fertility characteristics can not be assumed linear. For bovine sperm cells exposed to the flow characteristics described at 30 psi, sperm cell fertility

characteristics were substantially the same as nonsorted controls or comparable to nonsorted controls, and for a few responses better, if sperm cells are to be used for insemination or artificial insemination of females of the bovine species. Similar procedures were conducted with sperm cells obtained from stallions with similar results  
5 and conclusions.

Sperm cell fertility characteristics can be controlled and with respect to sperm cells obtained from mammals. For most species of mammals altering fluid stream characteristics to incrementally reduce fluid stream pressure, whether in the context of  
10 flow cytometry or otherwise, can result in a graded series of corresponding sperm cell samples having altered sperm cell fertility characteristics which may be used for a variety of procedures including artificial insemination, in vitro fertilization, or intracytoplasmic injection as described below.

The invention provides a alternate tests to assess binomial responses such as pregnant/not pregnant, which typically require large numbers of animals per treatment to obtain statistical significance unless treatment differences are fairly large. One embodiment of the invention which can amplify differences in sperm cell fertility characteristics of sex selected sperm due to treatment differences comprises competitive,  
20 or heterospermic, fertilization, mixing sperm of different treatments or males before insemination, and determining the proportion of embryos, fetuses or offspring derived from each male or treatment.

For example, fertility after sex selection of sperm cells by flow cytometry or by  
25 cell sorting for DNA content at two different fluid stream pressures can be assessed using heterospermic insemination using sex as the genetic marker. Sperm cells from each of two bulls was sorted into X-chromosome bearing or Y-chromosome populations, or both, at about 95% accuracy with the fluid stream pressure adjusted to either 30 psi or 50 psi. After concentrating sperm cells post-sort by centrifugation,  $1 \times 10^6$  X- chromosome  
30 bearing sperm cells sorted at 30 psi were placed in 0.25-mL straws with  $1 \times 10^6$  Y- chromosome bearing sperm cells sorted at 50 psi for each bull, as well as the converse in other straws:  $1 \times 10^6$  Y-sperm at 30 psi plus  $1 \times 10^6$  X-sperm at 50 psi. These sperm cells, along with unsorted controls, were then frozen, thawed some months later, and

inseminated into the body of the uterus of 85 Holstein heifers either 12 or 24 h after observed estrus with subgroups balanced across two inseminators.

Two months post-insemination, 81% of the 43 heifers becoming pregnant had  
5 fetuses of the sex (determined by ultrasound) corresponding to the sex of sperm processed at 30 psi. This differed from the 50:50 sex ratio expected ( $P < 0.01$ ), if there was no difference in sperm cell fertility characteristics of sperm cells sorted at the two pressures. The pregnancy rate with sex selected sperm at  $2 \times 10^6$  sperm per dose was 51% (43/85); this was similar to the controls of  $20 \times 10^6$  unsexed sperm per dose from the same  
10 ejaculates, 39% (9/23).

Another embodiment of the invention provides a method altering the cleavage rate and rate of blastocyst formation using sperm cells having controlled sperm cell fertility characteristics. Two bovine sperm cell samples each having controlled fertility  
15 characteristics were generated by flow sorting bovine sperm cells at 40 psi and 50 psi respectively. Dose response of sperm cell concentration in the fertilization medium was conducted with X-chromosome bearing sperm cells and Y-chromosome bearing sperm cells from each sperm cell sample. Thus, a multifactorial procedure comprising 2 fluid stream pressures, 3 sperm cell concentrations (1, 0.33 and  $0.11 \times 10^6$  sperm/ml), 6 bulls  
20 and 2 sexes can be conducted.

About 2,000 oocytes were aspirated from about 2mm to about 8mm follicles from slaughterhouse ovaries. Chemically defined media (CDM) were used throughout as described by Journal of Animal Sciences, 78:152-157 (2000), hereby incorporated by  
25 reference herein. Maturation took place in M-CDM supplemented with 0.5% FAF-BSA, 15 ng/ml NIDDK-oFSH-20, 1  $\mu$ g/ml USDA-LH-B-5, 0.1  $\mu$ g/ml  $E_2$ , 50 ng/ $\mu$ l EGF and 0.1 mM cysteamine for 23 h at 38.8°C and 5%  $CO_2$  in air. Sorted sperm cells frozen with  $2 \times 10^6$  cells per straw were thawed and centrifuged at 400 g through 2 ml 45% and 2 ml 90% Percoll gradients for 20 min. Then the supernatant was discarded and 2 ml of FCDM  
30 supplemented with 0.5% FAF-BSA, 2 mM caffeine and 0.02% heparin was added to the sperm pellet and centrifuged at 500 g for 5 min. The supernatant was discarded leaving approximately 50  $\mu$ l of sperm suspension. Matured oocytes were washed once in FCDM and transferred in groups of 15 in 5  $\mu$ l into 25- $\mu$ l drops of FCDM under mineral oil. Fertilization took place by adding 10  $\mu$ l of sperm suspension per drop for 18 h at 38.8°C,

5% CO<sub>2</sub> in air. Presumptive zygotes were cultured in CDM1 for 2 d and CDM2 for 4.5 d at 38.5°C, 5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub>. On day 7.5, blastocyst development was evaluated: Quality 1 to 4 (1 being excellent and 4 being poor) and stage of development, 6 to 8 (6 full, 6.5 expanding, 7 expanded, 7.5 hatching and 8 hatched blastocysts). Data (Table 1) were analyzed by ANOVA and first deviation after arc sin transformation.

Cleavage (53.6 and 43.6%) and blastocyst (18.2 and 14.7%) rates were higher for procedures utilizing sperm cells having controlled sperm cell characteristics obtained at about 40 psi than at about 50 psi (P<0.01). There was no interaction between dose and pressure; therefore, there was a similar advantage to lower pressure at each sperm concentration. A clear dose response of sperm cell concentration for cleavage and blastocyst production was found (Table 2). Also, there were large differences among bulls (P<0.01) for both responses, and there was a bull x dose interaction (P<0.01) for % cleaved. The data indicate that the sperm dose should be >1.0 x 10<sup>6</sup>/ml for some bulls. Embryo quality was higher (P<0.01) for Y-sperm than X-sperm (1.12 vs 1.57). Others have noted this for IVF embryos when embryos were sexed, and this effect now is confirmed with sexed sperm.

Table 2. Cleavage (% C) and blastocysts (% B) per oocyte data presented by bull.

| Sperm conc. (10 <sup>6</sup> ) | Bull |    |      |    |      |    |      |    |      |    |      |    |                 |                 |
|--------------------------------|------|----|------|----|------|----|------|----|------|----|------|----|-----------------|-----------------|
|                                | H023 |    | H024 |    | H025 |    | H026 |    | H027 |    | H028 |    | Avg             |                 |
|                                | C    | B  | C    | B  | C    | B  | C    | B  | C    | B  | C    | B  | C               | B               |
| 0.11                           | 18   | 1  | 6    | 1  | 4    | 11 | 36   | 15 | 20   | 7  | 54   | 15 | 30 <sup>a</sup> | 8 <sup>a</sup>  |
| 0.33                           | 44   | 4  | 7    | 2  | 72   | 31 | 62   | 21 | 29   | 11 | 68   | 18 | 47 <sup>b</sup> | 14 <sup>b</sup> |
| 1.0                            | 56   | 18 | 35   | 14 | 85   | 43 | 83   | 34 | 72   | 27 | 85   | 29 | 69 <sup>c</sup> | 28 <sup>c</sup> |

<sup>a,b,c</sup> Values without common superscripts within groups differ, P<0.01.

\*\*Fertility of sorted sperm has been low compared to unsorted control sperm, due partly to mechanical damage during sperm sorting by flow cytometry. Lowering system pressure improved both sperm quality and fertility in IVF. The present study evaluated the effect of system pressure during sperm sorting and extended maturation of oocytes on development

of embryos after ICSI. Sperm from each of 3 bulls were stained with 125  $\mu$ M Hoechst 33342 for 45 min at 34 °C, sorted into X- and Y-chromosome bearing populations at 95% accuracy with the pressure of SX MoFlo® sorters at 40 or 50 psi, and then cryopreserved. Fifty bovine oocytes obtained from slaughterhouse ovaries were placed per well with 1 ml of CDM1 supplemented with 0.5% FAF-BSA, 2mM glucose, 50 ng/ml EGF, 15 ng/ml NIDDK-oFSH-20, 1  $\mu$ g/ml USDA-LH-B-5, 1  $\mu$ g/ml E2 and 0.1 mM cysteamine, and then matured for 24h or 30h at 38.5°C, 5% CO<sub>2</sub> in air. Cumulus cells of matured oocytes were removed by vortexing, and oocytes with a polar body were selected. Motile sperm from sorted frozen-thawed semen were recovered by centrifugation through 2 ml each of 45 and 90% Percoll, and the concentration adjusted to  $4 \times 10^6$ /ml. Matured oocytes were divided into two injection groups, ICSI and sham injection using a Piezo injection system. The outer diameter of the sperm injection pipette was 8-10  $\mu$ m. All manipulations were performed at room temperature (24-25°C). After injection, oocytes were activated with 5  $\mu$ M ionomycin for 4 min, cultured in 50  $\mu$ l of CDM1 at 38.5°C under 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>, and assessed for cleavage at 72 h post-injection. Uncleaved oocytes from ICSI and sham injected groups were stained with orcein and evaluated for fertilization status. Cleaved embryos were further cultured and blastocyst development was evaluated on day 7.5 after injection. Data were subjected to ANOVA; the arc sin transformation was used for percentage data.

20

With 24h matured oocytes, there were no differences ( $P>0.1$ ) between sperm sorted at 40 versus 50 psi for cleavage or blastocyst rates, nor was there pressure x bull interaction. There were significant effects of bulls for all responses studied ( $P<0.05$ ). When injected with sperm sorted at 40 psi, oocytes matured for 30h resulted in a higher cleavage rate than 24h matured oocytes (22.9 versus 12.2%,  $P<0.05$ ), with no difference ( $P>0.1$ ) in blastocyst rate. Overall blastocyst development was higher in ICSI than in sham injection (7.5 versus 1.3%,  $P<0.05$ ). When uncleaved oocytes from 24h maturation were evaluated for fertilization status, ICSI showed higher percentage with 2 polar bodies and/or decondensed sperm compared to sham injection (15.7 versus 1.7%,  $P<0.05$ ). With 30h matured oocytes there was no difference in fertilization status between those two groups. We conclude that there was no difference in cleavage or development to blastocysts after ICSI using motile sperm that had been sorted at 40 vs 50 psi.

30

In another embodiment of the invention, heterospermic insemination using sex as the genetic marker can be used to rank fertility of males and to rank fertility of sperm treatments not involving sperm sexing. Current in vitro tests of sperm function are not highly correlated with male fertility, and homospermic inseminations require hundreds of  
5 inseminations per treatment to obtain accurate fertility data. Heterospermic insemination, mixing the sperm of two or more males, provides an accurate estimation of relative fertility in most species examined.

Frozen, flow-sorted sperm from 4 groups of 4 bulls were thawed and inseminated  
10 into heifers 12h or 24h following onset of estrus in all combinations of 3 bulls within groups (ABC, ABD, ACD, BCD). Equal numbers of progressively motile sperm were inseminated from each bull, totaling 600,000 motile sperm post-thaw. Half of each inseminate was deposited into each uterine horn. Embryos were collected nonsurgically 14.5 to 20 days following estrus. Collections yielded 165 elongating embryos from 332  
15 heifers (48%). Polymorphic DNA markers were used to genotype embryos to determine the sire of each embryo biopsy. After genotyping, 118 of the 165 embryos could be assigned a specific sire. Heterospermic indices for ranking each bull group were calculated using the maximum likelihood analysis theorem. Each bull within groups was ranked based on these indices (Table 1). In group 1, the fertility of the poorest bull was  
20 significantly lower ( $P<0.05$ ) than two other bulls. In group 2, the dominant bull had the highest index ( $P<0.05$ ). Similar distinctions could be made in groups 3 and 4. However, in three of the groups the fertility of some bulls was not clearly high or low ( $P>0.05$ ).

Table 1. Heterospermic indices  $\pm$  SE for individual bulls within groups.

| Group 1                       | Group 2                     | Group 3                       | Group 4                       |
|-------------------------------|-----------------------------|-------------------------------|-------------------------------|
| 1.47 $\pm$ .41 <sup>a</sup>   | 2.43 $\pm$ .43 <sup>a</sup> | 1.68 $\pm$ .44 <sup>a</sup>   | 0.92 $\pm$ .36 <sup>a,b</sup> |
| 0.44 $\pm$ .27 <sup>a,b</sup> | 0.22 $\pm$ .15 <sup>b</sup> | 1.09 $\pm$ .39 <sup>a,b</sup> | 0.46 $\pm$ .20 <sup>a</sup>   |
| 1.84 $\pm$ .46 <sup>a</sup>   | 0.90 $\pm$ .35 <sup>b</sup> | 0.83 $\pm$ .31 <sup>a,b</sup> | 2.02 $\pm$ .40 <sup>b</sup>   |
| 0.25 $\pm$ .17 <sup>b</sup>   | 0.45 $\pm$ .23 <sup>b</sup> | 0.40 $\pm$ .22 <sup>b</sup>   | 0.59 $\pm$ .24 <sup>a</sup>   |

25 <sup>a,b</sup> Indices without common superscripts differ,  $P<0.05$ .

With these procedures, an average of 30 genotyped embryos per group of 4 bulls enabled detection of bulls with clearly differing fertility. Sperm treatments also could be evaluated with this technique. This in vivo test requiring few females rapidly provides information concerning which bulls have relatively high or low fertility.

5

The population of calves obtained by artificial insemination of females with sex selected sperm cells in accordance with the invention are virtually identical to controls using unsex selected sperm cells. Furthermore, artificial insemination of females with sex selected sperm resulted in approximately 90% of calves of the planned sex. As described  
10 above, sperm cells can be sex selected on the basis of DNA content by flow cytometry or by cell sorting after staining with H33342. The sex selected sperm cells can then be cryopreserved as described in Theriogenology, 52:1375 (\_\_\_\_\_), hereby incorporated by reference herein.

15

Estrus can be synchronized in heifers and cows of various beef and dairy breeds, either by feeding 0.5 mg melengestrol acetate (MGA) daily for 14 d followed by 25 mg prostaglandin  $F_2$  ( $PGF_2$ ) im 17 to 19 d later or injection of 25 mg  $PGF_2$  im at 12-d intervals. Insemination with either frozen-thawed sex selected insemination samples or frozen-thawed sperm from the same ejaculate have been accomplished at either 12 hours  
20 or 24 hours after initial observation of estrus. For each breeding group, about 2/3 of the inseminations were with sexed sperm while control sperm were used in the remainder. Pregnancy and fetal sex were diagnosed by ultrasound 2 months later.

Cattle were managed at 13 farms through calving and weaning under differing  
25 levels of management (N = 49 to 228 per farm). Data collected included gestation length, birth weight, calving ease (1 = normal to 4 = Caesarian), weaning weight, neonatal deaths, and deaths from birth to weaning. Not all farms recorded birth and weaning weights. Data were subjected to factorial analysis of variance with factors: management groups, sorted versus control sperm, and sex of calves. The arc sin transformation was used for  
30 percentage data. Least-square means are in Table 1.

Table 1. Calving results from sexed and control calves

| Treatment          | N   | Gestation length (d) | Neonatal death (%) | Calving ease | Birth weight (kg) | Live weaning (%) | at Weaning weight (kg) |
|--------------------|-----|----------------------|--------------------|--------------|-------------------|------------------|------------------------|
| Sexed <sup>a</sup> | 574 | 279                  | 3.9                | 1.31         | 34.3              | 92.0             | 239                    |
| Control            | 385 | 279                  | 5.9                | 1.30         | 34.1              | 88.9             | 239                    |

<sup>a</sup> No significant differences (P>0.1) for any response.

There were no differences (P>0.1) between calves from sexed versus control groups for any response studied, nor were there significant interactions. There were significant effects of management groups for all responses studied (P<0.001 for all except % alive at weaning, P<0.02). Also, there were significant differences (all P<0.001) between female and male calves for birth weight: 32.2 and 35.5 kg; weaning weight: 232 and 246 kg; calving difficulty: 1.20 and 1.42; and gestation length: 278 and 280 d.

The sex ratio of the control calves was 51.0% males (N=382). X sort sperm resulted in 87.7% females, while the Y sort sperm produced 93.6% males (N=94). A few calves that were dead at birth did not have sex recorded and are not included.

The recent development of flow cytometric separation of stallion spermatozoa has resulted in the production of normal foals with preselected sex. (Lindsey A.C., Morris L.H., Allen W.R., Schenk J.L., Squires E.L., Bruemmer J.E. Equine vet. J. 2002, 34: 128 - 132). For this technology to be accessible, semen will be transported from the flow cytometer to the mare. This study examined the longevity and acrosome status of fresh stallion spermatozoa after sex preselection. Three ejaculates from each of 7 stallions were collected by artificial vagina and shipped to the laboratory at 20°C for 2-6h in a skim milk-glucose extender (1:1 v/v). The semen was centrifuged at 400g for 10min and the seminal plasma removed. The sperm pellet was resuspended to 100 x10<sup>6</sup>/ml in Kenneys modified Tyrodes medium (KMT), stained with Hoechst 33342 (5mg/ml, Sigma-Aldrich, St. Louis MO), incubated for 30min and subjected to flow cytometry. The sorted spermatozoa were centrifuged and resuspended to 40 x10<sup>6</sup>ml in KMT in 250µl aliquots for 48h storage at either 4°C or 20°C. The total progressive motility (TPM) and the acrosome status of the spermatozoa were evaluated prior to sorting and at 0, 2, 12, 24, 36 and 48h

after sorting. The TPM was evaluated microscopically and acrosomes stained with FITC-PNA (Sigma-Aldrich) and classified as intact, patchy or lost. The effect of stallions, time and storage temperatures were analyzed using the Proc GLM procedure and least means comparisons made (SAS Institute).

5

There was an effect of stallion ( $p = 0.03$ ) on sperm motility and on the proportion of intact acrosomes over time. Staining and incubating the spermatozoa with Hoechst 33342 resulted in a decrease in the proportion of intact acrosomes (Table 1). However, the proportion of intact acrosomes observed after sorting was higher than in the sperm population prior to sorting. The proportion of intact acrosomes declined ( $p < 0.0001$ ) as the lost acrosomes increased ( $p < 0.0001$ ) during 48h after sorting, but there was no effect of time on the proportion of patchy acrosomes. There was a significant effect of sperm storage temperature after sorting such that that storage for 12h at 20°C resulted in higher motility than storage at 4°C. Sex-sorting spermatozoa by flow cytometry results in the selection of a population of spermatozoa which can maintain acrosome integrity for 24h, equivalent to fresh spermatozoa. The maintenance of sperm longevity for 12h after FACS separation should enable sex-sorted spermatozoa to be shipped to mares located some distance from the site of the flow cytometer.

20 Table 1 The motility and acrosome status of flow sorted spermatozoa over time.

| Stage of Processing |           | TPM                      | Acrosome intact          |
|---------------------|-----------|--------------------------|--------------------------|
| Prestain            |           | 50.7 ± 10.2              | 57.1 ± 28.2              |
| Post stain          |           | 42.0 ± 17.1              | 42.3 ± 26.7              |
| 25 Post incubation  |           | 48.0 ± 15.7              | 34.9 ± 27.1 <sup>a</sup> |
| 0h                  | Post sort | 49.9 ± 18.3              | 60.2 ± 22.3 <sup>b</sup> |
| 2h                  | 4°C       | 34.3 ± 19.3              | 47.1 ± 28.3              |
|                     | 20°C      | 40.8 ± 21.8              | 53.9 ± 24.5              |
| 12h                 | 4°C       | 8.5 ± 12.8 <sup>a</sup>  | 59.8 ± 19.7              |
| 30                  | 20°C      | 27.0 ± 21.0 <sup>b</sup> | 66.6 ± 12.0              |
| 24h                 | 4°C       | 4.8 ± 12.9               | 56.2 ± 16.8              |
|                     | 20°C      | 17.2 ± 17.9              | 64.3 ± 16.4              |
| 48h                 | 4°C       | 0.0 ± 0.0                | 32.7 ± 25.0              |
| 35                  | 20°C      | 5.1 ± 8.6                | 41.6 ± 24.0              |

<sup>a,b</sup>Values within a column with different superscripts are significantly different ( $p < 0.05$ ).

Foals of predetermined sex have been accurately and reliably produced in a research setting (Lindsey et al., Equine Vet. J. 2002, 34: 128-132). Sex-sorted sperm would be more efficiently utilized by the industry, however, if frozen/thawed sex-sorted sperm were available. The objective of this study was to compare the motion characteristics of sperm

that had been stored for 18h at 15°C, flow-sorted, and then frozen, to sperm that had been cryopreserved immediately following shipment at 18h at 15°C. Two ejaculates were used from each of five stallions. Following collection, sperm for both treatments were extended to 25x10<sup>6</sup>/mL in a Kenney + Modified Tyrodes (KMT) medium and stored in a water bath at 15°C for 18 h. After storage, sperm were allowed to reach ambient temperature (~22°C) prior to centrifugation at 600g for 10 min. Seminal plasma was removed and the sperm pellet resuspended to 500 x 10<sup>6</sup>/ml in KMT. An aliquot of sperm was removed (Control) from this sample, extended to 87 x 10<sup>6</sup>/ml in a skim-milk, egg yolk freezing extended (4% glycerol; FR5), and allowed to slow cool to 5°C for 90 min before freezing in liquid nitrogen vapor. A second aliquot (Flow-sorted) of sperm was extended to 100 x 10<sup>6</sup>/ml in KMT, stained with Hoechst 33342 (5mg/ml, Sigma-Aldrich, St. Louis MO), incubated for 30 minutes, and subsequently sorted by flow-cytometry. Sorted sperm was centrifuged at 850g for 20 min, resuspended to 87 x 10<sup>6</sup>/ml in FR5, and allowed to cool slowly to 5°C for 90 min prior to cryopreservation. Sperm for both treatments were packaged in 0.25-ml straws, and each straw contained 20 million sperm. Sperm were evaluated (blindly) for visual progressive motility (2 observers) at 30 and 90 min post-thaw. An aliquot of sperm from each straw was diluted in both KMT and in KMT containing 2mM caffeine. Samples were allowed to equilibrate for 5-10 min at 37°C prior to evaluation. A second straw of each treatment was evaluated (with and without caffeine) using the Hamilton-Thorn Motility Analyzer (CASA). Results are in Table 1. Differences in motion parameters were determined by ANOVA. According to most measured responses, flow-sorting was detrimental to sperm motility. Additionally, 2 mM caffeine improved many sperm responses. There was an interaction whereby caffeine improved some responses more for sorted sperm than for control sperm. Therefore, the damage caused by sorting can be partially compensated for by caffeine. It is possible that similar compensation may occur in the mare reproductive tract. Studies are currently in progress to compare the fertility of stored, cryopreserved stallion sperm to that of sperm that has been stored and sorted prior to cryopreservation.

| Treatment | Vis             | Vis             | CASA            | CASA            | VAP             | VSL             | VCL              | ALH               | BCF             | STR             | LIN             |
|-----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|------------------|-------------------|-----------------|-----------------|-----------------|
|           | 30              | 90              | Tot             | Prog            |                 |                 |                  |                   |                 |                 |                 |
| C-Control | 50 <sup>a</sup> | 47 <sup>a</sup> | 64 <sup>a</sup> | 60 <sup>a</sup> | 94 <sup>a</sup> | 80 <sup>a</sup> | 164 <sup>a</sup> | 6.19 <sup>a</sup> | 33 <sup>a</sup> | 83 <sup>a</sup> | 50 <sup>a</sup> |
| Control   | 45 <sup>a</sup> | 40 <sup>b</sup> | 50 <sup>b</sup> | 44 <sup>b</sup> | 82 <sup>a</sup> | 69 <sup>a</sup> | 144 <sup>a</sup> | 5.73 <sup>a</sup> | 33 <sup>a</sup> | 82 <sup>a</sup> | 50 <sup>a</sup> |
| C-Sorted  | 32 <sup>b</sup> | 31 <sup>c</sup> | 32 <sup>c</sup> | 21 <sup>c</sup> | 48 <sup>b</sup> | 38 <sup>b</sup> | 98 <sup>b</sup>  | 4.75 <sup>b</sup> | 41 <sup>b</sup> | 73 <sup>b</sup> | 39 <sup>b</sup> |
| Sorted    | 18 <sup>c</sup> | 16 <sup>d</sup> | 24 <sup>d</sup> | 12 <sup>d</sup> | 39 <sup>b</sup> | 30 <sup>b</sup> | 80 <sup>b</sup>  | 4.51 <sup>b</sup> | 37 <sup>c</sup> | 69 <sup>b</sup> | 38 <sup>b</sup> |

<sup>a, b, c, d</sup>Values in the same column without common superscripts differ (P,0.05).  
C-treatments stimulated with 2mM caffeine.

Cow elk 3-6-yr of age in Colorado and Minnesota were synchronized for estrus in September by insertion of a progesterone CIDR into the vagina for 12-14 d. Upon  
5 removal of the CIDR, 200 IU of eCG was administered intramuscular and elk were timed-inseminated 60 h later. Fresh semen was collected via electro-ejaculation from a 5-yr old bull elk and slowly cooled over 4 h to about 20°C for transportation as a neat ejaculate to the sperm-sorting laboratory. The ejaculate was concentrated to  $1 \times 10^9$  sperm/ml for straining by centrifuging 1.5 ml aliquots for 10 sec at 15,000 x g. Semen was incubated in  
10 112  $\mu$ M Hoechst 33342 at  $200 \times 10^6$  sperm/ml in a TALP medium for 45 min at 34°C, and then diluted to  $100 \times 10^6$ /ml for sorting. Sperm were sorted on the basis of differing DNA content of X and Y chromosome-bearing sperm. X chromosome-bearing elk sperm contained 3.8% more DNA than Y chromosome-bearing sperm. Sperm were flow-sorted over a 4 h period using MoFlo@SX operating at 50 psi with a TRIS-based sheath fluid.  
15 The 351 and 364 bands of an argon laser, emitting 150 mW, excited Hoechst 33342 dye bound to DNA. Both X and Y chromosome-bearing sperm were collected (~92% purity as verified by reanalyzing sonicated sperm aliquots for DNA) were collected at ~4,700 sperm/sec into tubes containing 2 ml of 20% egg yolk-TRIS extender. Sorted volumes of 15 ml were sequentially collected. Approximately  $110 \times 10^6$  sperm of each sex were  
20 sorted and cooled to 5 °C over 90 min. An equal volume of glycerol (12%) containing extender was added to the sorted volume at 5 °C. Sorted sperm aliquots containing 30-ml were concentrated by centrifugation at 4 °C for 20 min at 850 x g. Sperm pellets were pooled, adjusted to  $21.7 \times 10^6$  sperm/ml and loaded into 0.25-ml straws. Each straw, containing  $5 \times 10^6$  total sperm, was frozen in liquid nitrogen vapor. As a control,  $5 \times 10^6$   
25 total sperm from the same ejaculate were frozen in 0.25 ml straws at the same time as the sexed sperm. After thawing for 30 sec at 37 °C, 65% and 60% of sperm (control and sexed, respectively) were progressively motile as determined by visual estimates. Cows at 3 different locations and management schemes were inseminated using routine trans-cervical semen deposition in the uterine body. Pregnancy was determined 40-d post  
30 insemination by assaying blood for Pregnancy-Specific Protein B (Bio Tracking, Moscow, Idaho). Ten cows at one location were in poor condition at the time of insemination and no pregnancies were achieved with sexed or control sperm. The pregnancy rate at the other locations with sexed sperm (61%; 11/18) was similar to that for control inseminates (50%; 3/6). These pregnancy rates (sexed and controls) resulted from fewer sperm than

are used in normal elk artificial insemination. Nine of eleven (82%) of sexed calves were of the predicted sex.

The invention can further include a mammal produced in accordance with any of  
5 the above described embodiments of the invention, or can include a mammal of  
predetermined sex in accordance with the various embodiments of the invention that  
provide sperm cell insemination samples having an enriched population of either X-  
chromosome bearing sperm cells or enriched population of Y-chromosome bearing sperm  
cells, or a mammal produced in accordance with any embodiment of the invention in  
10 which a sperm cell insemination sample containing a low number of sperm cells compared  
to the typical number used to inseminate that particular species of mammal is used, or elk  
progeny produced in accordance with the invention as described above.

As can be easily understood from the foregoing, the basic concepts of the present  
15 invention may be embodied in a variety of ways. It involves both a sperm cell process  
system including both techniques as well as devices to accomplish sperm cell processing.  
In this application, various sperm cell processing techniques 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  
20 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. Importantly, as to all of the foregoing, all of these facets should be  
understood to be encompassed by this disclosure.

25 The discussion included in this nonprovisional application is intended to serve as a  
basic description. The reader should be aware that the 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  
30 of alternative or equivalent elements. Again, these are implicitly included in this  
disclosure. Where the invention is described in device-oriented terminology, each element  
of the device implicitly performs a function. Apparatus claims may not only be included  
for the device 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 will be included in a full patent application.

It should also be understood that a variety of changes may be made without departing from the essence of the invention. Such changes are also implicitly included in the description. They still fall within the scope of this invention. A broad disclosure encompassing both the explicit embodiment(s) shown, the great variety of implicit alternative embodiments, and the broad methods or processes and the like are encompassed by this disclosure.

10

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 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 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 "flow-sorter" should be understood to encompass disclosure of the act of "flow-sorting" -- whether explicitly discussed or not -- and, conversely, were there effectively disclosure of the act of "flow-sorting", such a disclosure should be understood to encompass disclosure of a "flow-sorter" and even a "means for flow-sorting". Such changes and alternative terms are to be understood to be explicitly included in the description.

30

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. 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. Finally, all references listed in the list of References To Be Incorporated By  
5 Reference In Accordance With The Provisional Patent Application or other information statement filed with the application are hereby appended and 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  
10 the applicant(s).

Thus, the applicant(s) should be understood to claim at least: i) each of the sperm cell processing devices as herein disclosed and described, ii) the related methods disclosed and described, iii) similar, equivalent, and even implicit variations of each of these devices  
15 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 enhanced by the various systems or components disclosed,  
20 viii) the resulting products produced by such systems or components, and ix) methods and apparatuses substantially as described hereinbefore and with reference to any of the accompanying examples, x) the various combinations and permutations of each of the elements disclosed, and xi) each potentially dependent claim or concept as a dependency on each and every one of the independent claims or concepts presented. In this regard it  
25 should be understood that for practical reasons and so as to avoid adding potentially hundreds of claims, the applicant may eventually present claims with initial dependencies only. Support should be understood to exist to the degree required under new matter laws -- including but not limited to European Patent Convention Article 123(2) and United States Patent Law 35 USC 132 or other such laws-- to permit the addition of any of the  
30 various dependencies or other elements presented under one independent claim or concept as dependencies or elements under any other independent claim or concept. Further, if or when used, the use of the transitional phrase "comprising" is used to maintain the "open-end" claims herein, according to traditional claim interpretation. Thus, 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.

5

## VI. CLAIMS

We claim:

1. A method of generating a sperm cell insemination sample, comprising:
  - a. obtaining semen from a male of a species of mammal;
  - b. generating a fluid stream having flow characteristics;
  - c. altering flow characteristics of said fluid stream to adjust fluid stream pressure;
  - d. entraining said sperm cells into said fluid stream;
  - e. controlling sperm cell fertility characteristics through adjustment of said fluid stream pressure; and
  - f. generating a sperm cell insemination sample having controlled sperm cell fertility characteristics.
2. A method of generating a sperm cell insemination sample as described in claim 1, where in said species of mammal is selected from the group consisting of a bovine species of mammal, an equine species of mammal, an ovine species of mammal, a canine species of mammal, a feline species of mammal, a swine species of mammal, a marine species of mammal, a deer species of mammal, a primate species of mammal, a goat species of mammal.
3. A method of generating a sperm cell insemination sample as described in claim 1, wherein said fluid stream comprises a sheath fluid stream.
4. A method of generating a sperm cell insemination sample as described in claim 3, wherein said sheath fluid stream comprises a sheath fluid containing phosphate buffered saline (PBS).
5. A method of generating a sperm cell insemination sample as described in claim 3, wherein said sheath fluid stream comprises a sheath fluid containing a citrate buffer.
6. A method of generating a sperm cell insemination sample as described in claim 5, wherein said citrate buffer comprises about 2.9% sodium citrate.

7. A method of generating a sperm cell insemination sample a described in claim 3, wherein said sheath fluid stream comprises a sheath fluid containing a HEPES buffer.
8. A method of generating a sperm cell insemination sample a described in claim 1,  
5 wherein said fluid stream is generated within a flow cytometer or cell sorter.
9. A method of generating a sperm cell insemination sample a described in claim 1, wherein said step of altering flow characteristics of said fluid stream to adjust fluid stream pressure comprises altering said flow characteristics to adjust said fluid stream pressure to  
10 between about 20 pounds per square inch and about 60 pounds per square inch.
10. A method of generating a sperm cell insemination sample a described in claim 1, wherein said step of obtaining semen from a male of a species of mammal comprises obtaining bovine semen from said male of a bovine species of mammal and said step of  
15 controlling sperm cell fertility characteristics through adjustment of said fluid stream pressure comprises establishing bovine sperm cell fertility characteristics of bovine sperm cells entrained within said fluid stream which are not substantially different than said bovine sperm cell fertility characteristics of said bovine sperm cells in said semen.
- 20 11. A method of generating a sperm cell insemination sample a described in claim 1, wherein said step of obtaining semen from a male of a species of mammal comprises obtaining bovine semen from said male of a bovine species of mammal and said step of controlling sperm cell fertility characteristics through adjustment of said fluid stream pressure comprises establishing said bovine sperm cell fertility characteristics of said  
25 bovine sperm cells entrained within said fluid stream which are substantially comparable said bovine sperm cell fertility characteristics of said bovine sperm cells in said semen.
12. A method of generating a sperm cell insemination sample a described in claim 1, wherein said step of obtaining semen from a male of a species of mammal comprises  
30 obtaining said bovine semen from said male of a bovine species of mammal and said step of generating a sperm cell insemination sample having controlled sperm cell fertility characteristics comprises generating a bovine sperm cell insemination sample having said bovine sperm cell fertility characteristics which are not substantially different than bovine sperm cell fertility characteristics of said bovine sperm cells in said bovine semen.

13. A method of generating a sperm cell insemination sample a described in claim 1, wherein said step of obtaining semen from a male of a species of mammal comprises obtaining said bovine semen from said male of a bovine species of mammal and said step  
5 of generating a sperm cell insemination sample having controlled sperm cell fertility characteristics comprises generating a bovine sperm cell insemination sample having bovine sperm cell fertility characteristics which are substantially comparable to bovine sperm cell fertility characteristics of said bovine sperm cells in said bovine semen.

10 14. A method of generating a sperm cell insemination sample a described in claim 1, wherein said step of obtaining semen from a male of a species of mammal comprises obtaining said bovine semen from said male of a bovine species of mammal and said step of altering flow characteristics of said fluid stream to adjust fluid stream pressure  
15 comprises adjusting said flow characteristics of said fluid stream to adjust fluid stream pressure to between about 30 pounds per square inch and about 50 pounds per square inch.

15. A method of generating a sperm cell insemination sample a described in claim 1, wherein said step of obtaining semen from a male of a species of mammal comprises obtaining said bovine semen from said male of a bovine species of mammal and said step  
20 of altering flow characteristics of said fluid stream to adjust fluid stream pressure comprises adjusting said flow characteristics of said fluid stream to adjust fluid stream pressure to between about 30 pounds per square inch and about 40 pounds per square inch.

16. A method of generating a sperm cell insemination sample a described in claim 1,  
25 wherein said step of obtaining semen from a male of a species of mammal comprises obtaining said bovine semen from said male of a bovine species of mammal and said step of altering flow characteristics of said fluid stream to adjust fluid stream pressure comprises adjusting said flow characteristics of said fluid stream to adjust fluid stream pressure to about 40 pounds per square inch.

30

17. A method of generating a sperm cell insemination sample a described in claim 1, wherein said step of obtaining semen from a male of a species of mammal comprises obtaining equine semen from said male of a equine species of mammal and said step of controlling sperm cell fertility characteristics through adjustment of said fluid stream

pressure comprises establishing equine sperm cell fertility characteristics of equine sperm cells entrained within said fluid stream which are not substantially different than said equine sperm cell fertility characteristics of said equine sperm cells in said semen.

- 5 18. A method of generating a sperm cell insemination sample a described in claim 1, wherein said step of obtaining semen from a male of a species of mammal comprises obtaining equine semen from said male of a equine species of mammal and said step of controlling sperm cell fertility characteristics through adjustment of said fluid stream pressure comprises establishing said equine sperm cell fertility characteristics of said  
10 equine sperm cells entrained within said fluid stream which are substantially comparable said equine sperm cell fertility characteristics of said equine sperm cells in said semen.

19. A method of generating a sperm cell insemination sample a described in claim 1, wherein said step of obtaining semen from a male of a species of mammal comprises  
15 obtaining said equine semen from said male of a equine species of mammal and said step of generating a sperm cell insemination sample having controlled sperm cell fertility characteristics comprises generating a equine sperm cell insemination sample having said equine sperm cell fertility characteristics which are not substantially different than equine sperm cell fertility characteristics of said equine sperm cells in said equine semen.

20

20. A method of generating a sperm cell insemination sample a described in claim 1, wherein said step of obtaining semen from a male of a species of mammal comprises obtaining said equine semen from said male of a equine species of mammal and said step of generating a sperm cell insemination sample having controlled sperm cell fertility  
25 characteristics comprises generating a equine sperm cell insemination sample having equine sperm cell fertility characteristics which are substantially comparable to equine sperm cell fertility characteristics of said equine sperm cells in said equine semen.

21. A method of generating a sperm cell insemination sample a described in claim 1,  
30 wherein said step of obtaining semen from a male of a species of mammal comprises obtaining said equine semen from said male of a equine species of mammal and said step of altering flow characteristics of said fluid stream to adjust fluid stream pressure comprises adjusting said flow characteristics of said fluid stream to adjust fluid stream pressure to between about 30 pounds per square inch and about 50 pounds per square inch.

22. A method of generating a sperm cell insemination sample a described in claim 1, wherein said step of obtaining semen from a male of a species of mammal comprises obtaining said equine semen from said male of a equine species of mammal and said step  
5 of altering flow characteristics of said fluid stream to adjust fluid stream pressure comprises adjusting said flow characteristics of said fluid stream to adjust fluid stream pressure to between about 30 pounds per square inch and about 40 pounds per square inch.

23. A method of generating a sperm cell insemination sample a described in claim 1,  
10 wherein said step of obtaining semen from a male of a species of mammal comprises obtaining said equine semen from said male of a equine species of mammal and said step of altering flow characteristics of said fluid stream to adjust fluid stream pressure comprises adjusting said flow characteristics of said fluid stream to adjust fluid stream pressure to about 40 pounds per square inch.

15 24. A method of generating a sperm cell insemination sample a described in claim 1, wherein one of said sperm cell fertility characteristics comprises sperm cell motility.

25. A method of generating a sperm cell insemination sample a described in claim 1,  
20 wherein said fertility characteristics comprises bovine sperm cell motility.

26. A method of generating a sperm cell insemination sample a described in claim 1, wherein said fertility characteristics comprises equine sperm cell motility.

25 27. A method of generating a sperm cell insemination sample a described in claim 1, wherein said fertility characteristics comprises sperm cell viability.

28. A method of generating a sperm cell insemination sample a described in claim 1, wherein said fertility characteristics comprises bovine sperm cell viability.

30 29. A method of generating a sperm cell insemination sample a described in claim 1, wherein said fertility characteristics comprises equine sperm cell viability.

30. A method of generating a sperm cell insemination sample a described in claim 1, wherein said fertility characteristics comprises pregnancy rate of a female of said species of mammal inseminated with said sperm cell insemination sample having controlled sperm cell fertility characteristics.

5

31. A method of generating a sperm cell insemination sample a described in claim 1, wherein said fertility characteristics comprises pregnancy rate of said female of said bovine species of mammal inseminated with a bovine sperm cell insemination sample having controlled sperm cell fertility characteristics.

10

32. A method of generating a sperm cell insemination sample a described in claim 1, wherein said fertility characteristics comprises pregnancy rate of said female of said equine species of mammal inseminated with an equine sperm cell insemination sample having controlled sperm cell fertility characteristics.

15

33. A method of generating a sperm cell insemination sample a described in claim 1, wherein said fertility characteristics comprises cleavage rate of oocytes fertilized with said sperm cell insemination sample having controlled sperm cell fertility characteristics.

20

34. A method of generating a sperm cell insemination sample a described in claim 1, wherein said fertility characteristics comprises cleavage rate of bovine oocytes fertilized with said bovine sperm cell insemination sample having controlled sperm cell fertility characteristics.

25

35. A method of generating a sperm cell insemination sample a described in claim 1, wherein said fertility characteristics comprises cleavage rate of equine oocytes fertilized with said equine sperm cell insemination sample having controlled sperm cell fertility characteristics.

30

36. A method of generating a sperm cell insemination sample a described in claim 1, wherein said fertility characteristics comprises blastocyst rate of oocytes fertilized with said sperm cell insemination sample having controlled sperm cell fertility characteristics.

37. A method of generating a sperm cell insemination sample a described in claim 1, wherein said fertility characteristics comprises blastocyst rate of bovine oocytes fertilized with said bovine sperm cell insemination sample having controlled sperm cell fertility characteristics.

5

38. A method of generating a sperm cell insemination sample a described in claim 1, wherein said fertility characteristics comprises blastocyst rate of equine oocytes fertilized with said equine sperm cell insemination sample having controlled sperm cell fertility characteristics.

10

39. A method of generating a sperm cell insemination sample a described in claim 31, wherein said bovine sperm cell insemination sample having controlled sperm cell fertility characteristics contains between about  $1 \times 10^5$  and  $2 \times 10^7$  of said bovine sperm cells.

15

40. A method of generating a sperm cell insemination sample a described in claim 31, wherein said bovine sperm cell insemination sample having controlled sperm cell fertility characteristics contains between about  $1 \times 10^6$  and  $3 \times 10^6$  of said bovine sperm cells.

41. A method of generating a sperm cell insemination sample a described in claim 1,  
20 further comprising the step of staining said sperm cells.

42. A method of generating a sperm cell insemination sample a described in claim 41, wherein said step of staining said sperm cells comprises staining said sperm cells with Hoechst 33342.

25

43. A method of generating a sperm cell insemination sample a described in claim 1, further comprising generating droplets in said fluid stream some of which contain one each of said sperm cells.

30 44. A method of generating a sperm cell insemination sample a described in claim 43, further comprising differentiating said sperm cells based upon a sex characteristic.

45. A method of generating a sperm cell insemination sample a described in claim 44, wherein said step of differentiating said sperm cells based on a sex characteristic comprises differentiating said sperm cells based on amount of DNA content.
- 5 46. A method of generating a sperm cell insemination sample a described in claim 44, wherein said step of differentiating said sperm cells based on a sex characteristic comprises differentiating said sperm cells based on sperm head volume.
47. A method of generating a sperm cell insemination sample a described in claim 44,  
10 further comprising the step of separating s sperm cells based on said sex characteristic.
48. A method of generating a sperm cell insemination sample a described in claim 47, further comprising the step of collecting said sperm cell insemination sample having controlled sperm cell fertility characteristics in a collection container.  
15
49. A method of generating a sperm cell insemination sample a described in claim 48, wherein said step of collecting said sperm cell insemination sample having controlled sperm cell fertility characteristics in a collection container comprises collecting a sex selected sperm cell insemination sample in said collection container.  
20
50. A method of generating a sperm cell insemination sample a described in claim 49, wherein said sex selected sperm cell insemination sample comprises an artificial insemination sex selected sperm cell insemination sample.
- 25 51. A method of generating a sperm cell insemination sample a described in claim 49, wherein said sex selected sperm cell insemination sample comprises an in vitro fertilization sex selected sperm cell insemination sample.
52. A method of generating a sperm cell insemination sample a described in claim 49,  
30 wherein said sex selected sperm cell insemination sample comprises an intracytoplasmic sex selected sperm cell injection sample.
53. A method of assessing sex selected sperm cell fertility, comprising:  
a. obtaining sperm cells from a male of a species of mammal;

- b. providing Y-chromosome bearing sex selected sperm cells exposed to first treatment conditions;
- c. providing X-chromosome bearing sex selected sperm cells exposed to second treatment conditions;
- 5 d. generating an insemination sample having a ratio of said Y-chromosome bearing sex selected sperm cells exposed to said first treatment conditions and said X-chromosome bearing sex selected sperm cells exposed to second treatment conditions;
- 10 e. inseminating at least one female of said species of mammal with said insemination sample having said ratio of said Y-chromosome bearing sperm cells exposed to said first treatment conditions and said X-chromosome bearing sperm cells exposed to second treatment conditions;
- f. producing offspring from said at least one female of said species of mammal;
- 15 g. assessing fertility of said sex selected sperm cells by comparison of sex ratio of offspring of said at least one female of said species of mammal to said ratio of said Y-chromosome bearing sperm cells exposed to first treatment conditions and X-chromosome bearing sperm cells exposed to second treatment conditions

20

54. A method of assessing sex selected sperm cell fertility as described in claim 53, wherein said ratio of said Y-chromosome bearing sperm cells exposed to said first treatment conditions and said X-chromosome bearing sperm cells exposed to second treatment conditions comprises substantially equal numbers of Y-chromosome bearing sperm cells exposed to said first treatment conditions and said X-chromosome bearing sperm cells exposed to second treatment conditions.

25

55. A method of assessing sex selected sperm cell fertility as described in claim 53, wherein said sex ratio of offspring of said at least one female of said species of mammal comprises a sex ratio of embryos.

30

56. A method of assessing sex selected sperm cell fertility as described in claim 53, wherein said sex ratio of offspring of said at least one female of said species of mammal comprises a sex ratio of fetuses.

56. A method of assessing sex selected sperm cell fertility as described in claim 53, wherein said sex ratio of offspring of said at least one female of said species of mammal comprises a sex ratio of offspring born.

5

57. A method of assessing sex selected sperm cell fertility as described in claim 53, wherein said at least one female of said species of said mammal comprises a sufficient number of females to of said species to produce sufficient numbers of offspring to establish said sex ratio of offspring.

10

58. A method of assessing fertility of sperm cells from a male of a species of mammal, comprising:

- a. obtaining sperm cells from a least two males of a species of mammal;
- b. exposing said sperm cells from each of said at least two males of said species to substantially the same flow cytometric treatment;
- c. inseminating at least one female of said species of mammal with a mixture of substantially equal numbers of said sperm cells from each of said at least two males of said species of mammal exposed to substantially the same flow cytometric treatment;
- d. collecting embryos from said at least one female of said species of mammal;
- e. determining which of said at least two males of said species of mammal sired each embryo; and
- f. ranking comparative fertility of said at least to males of said species of mammal based on comparative number of embryos sired by each of said at least two males.

20

59. A method of assessing fertility of sperm cells from a male of a species of mammal as described in claim 58, wherein said flow cytometric treatment comprises separating said sperm cells from each of said at least two males based upon a sex characteristic.

30

60. A method of assessing fertility of sperm cells from a male of a species of mammal as described in claim 58, wherein said flow cytometric treatment comprises sex selecting sperm cells from each of said at least two males.

61. A method of assessing fertility of sperm cells from a male of a species of mammal, comprising:

- a. obtaining sperm cells from a least two males of a species of mammal;
- 5 b. exposing said sperm cells from each of said at least two males of said species to substantially the same flow cytometric treatment;
- c. fertilizing oocytes in vitro with a mixture of substantially equal numbers of said sperm cells from each of said at least two males of said species of mammal exposed to substantially the same flow cytometric treatment;
- 10 d. collecting embryos generated through said in vitro fertilization of said oocytes;
- e. determining which of said at least two males of said species of mammal sired each embryo; and
- f. ranking comparative fertility of said at least two males of said species of mammal based on comparative number of embryos sired by each of said at  
15 least two males.

20

1/2

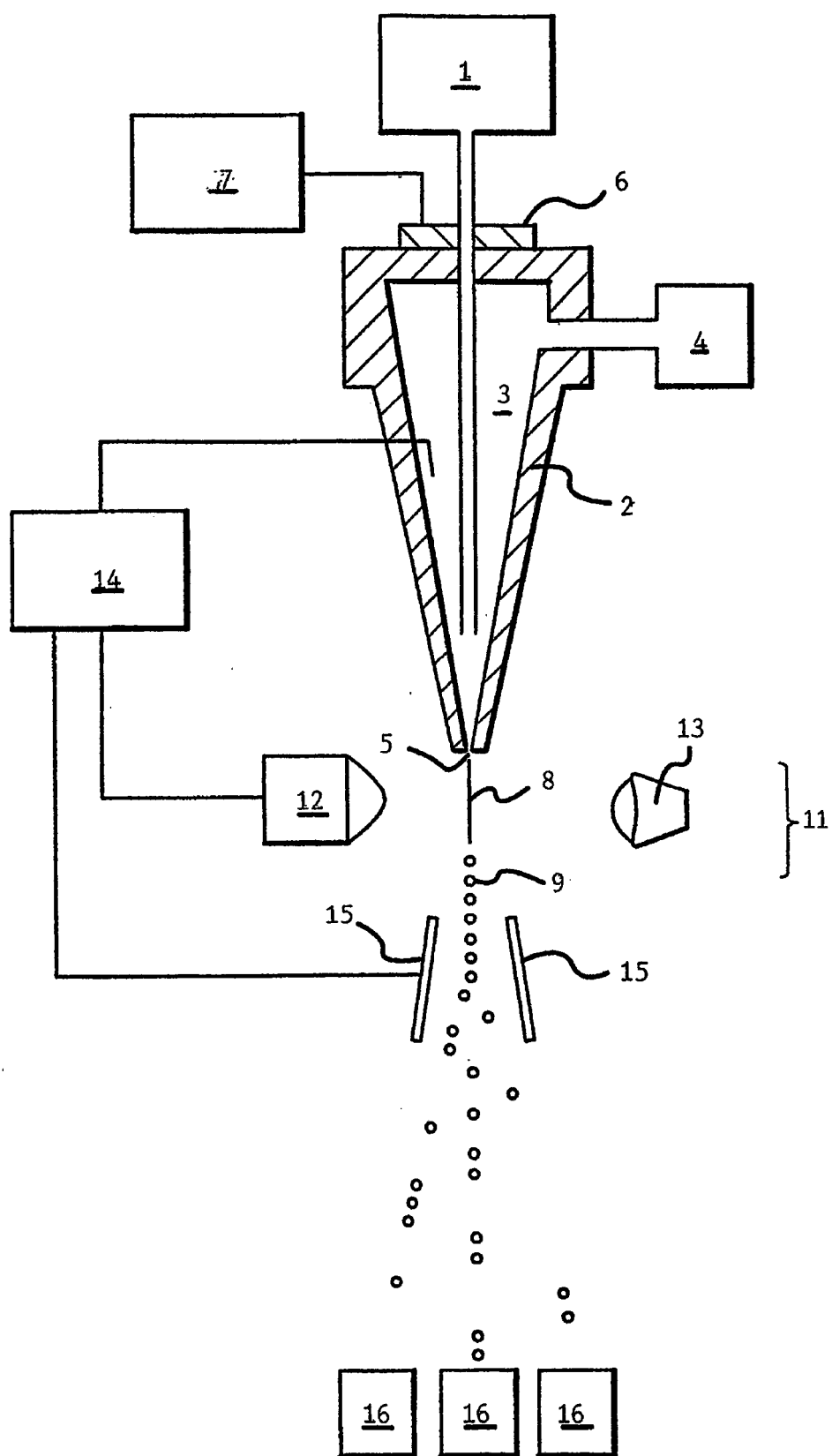


FIG.1

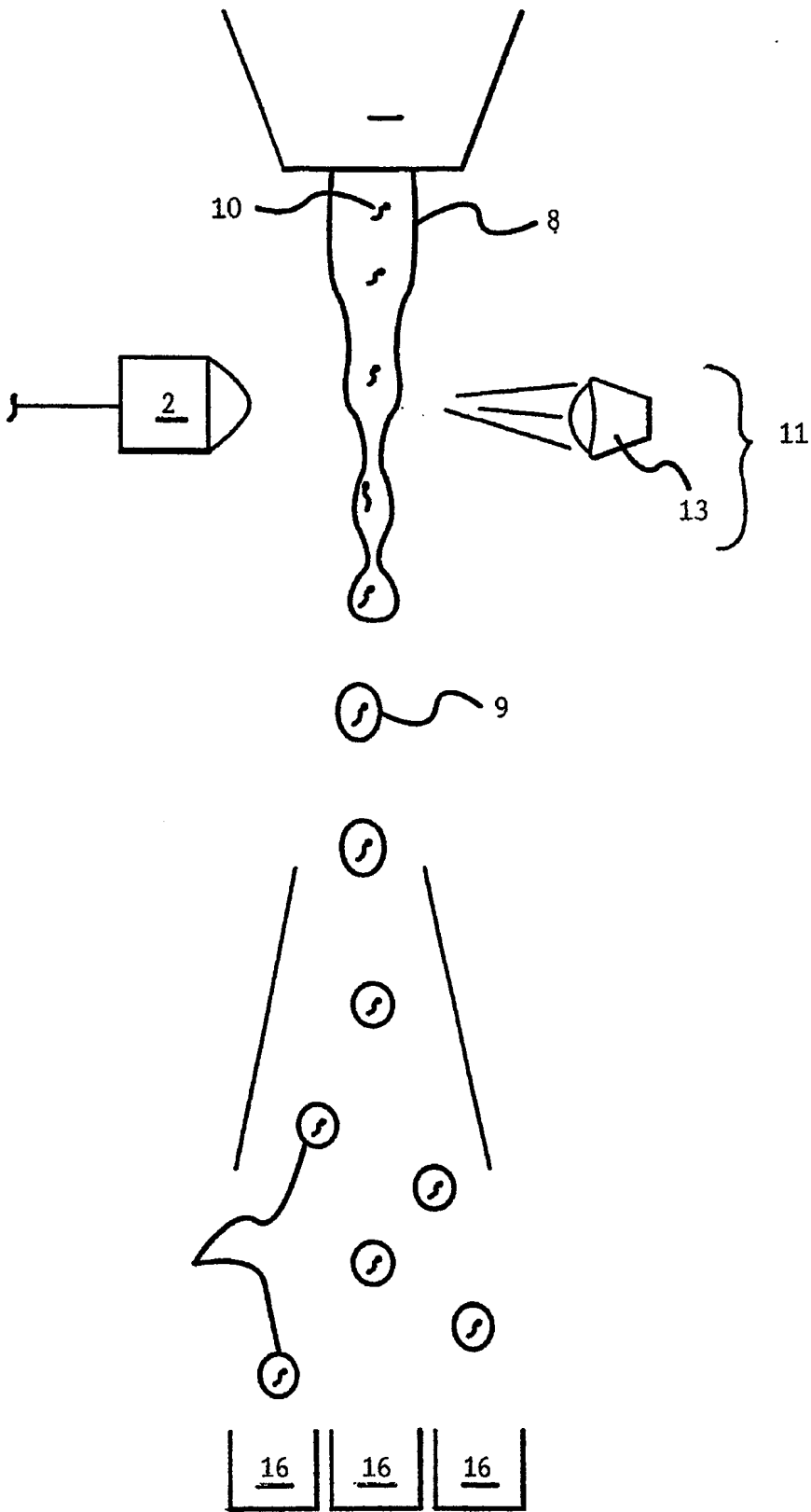


FIG.2

**ANEXO 5. COPIA DE LA PRIMERA PÁGINA DE LA PUBLICACIÓN DE PATENTES  
EN PARALELAS PARA ESTADOS UNIDOS US 7,713,687 Y US 7,771,921,  
ARGENTINA AR 036869B1 Y AUSTRALIA 2002237689, 2008200**



US007713687B2

(12) **United States Patent**  
Seidel et al.

(10) **Patent No.:** US 7,713,687 B2  
(45) **Date of Patent:** May 11, 2010

- (54) **SYSTEM TO SEPARATE FROZEN-THAWED SPERMATOOZOA INTO X-CHROMOSOME BEARING AND Y-CHROMOSOME BEARING POPULATIONS**
- (75) **Inventors:** George E. Seidel, LaPorte, CO (US); Kehuan Lu, Guangxi (CN); Tae Kwang Suh, Fort Collins, CO (US); David G. Cran, Aberdeen (GB)
- (73) **Assignee:** XY, Inc., Navasota, TX (US)
- (\*) **Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 173 days.
- (21) **Appl. No.:** 10/433,183
- (22) **PCT Filed:** Nov. 29, 2001
- (86) **PCT No.:** PCT/US01/45023  
§ 371 (c)(1),  
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- (87) **PCT Pub. No.:** WO02/43574  
**PCT Pub. Date:** Jun. 6, 2002

(65) **Prior Publication Data**  
US 2004/0049801 A1 Mar. 11, 2004

**Related U.S. Application Data**  
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(51) **Int. Cl.**  
A01N 1/02 (2006.01)  
C12Q 1/68 (2006.01)

(52) **U.S. Cl.** 435/2; 435/6

(58) **Field of Classification Search** None  
See application file for complete search history.

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(Continued)

**Primary Examiner**—Manjunath Rao  
**Assistant Examiner**—Paul C. Martin  
(74) **Attorney, Agent, or Firm**—Cindee Ewell; Ryan Christensen

(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 mammal. 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.

18 Claims, 3 Drawing Sheets



US007771921B2

(12) **United States Patent**  
**Seidel et al.**

(10) **Patent No.:** **US 7,771,921 B2**  
(45) **Date of Patent:** **\*Aug. 10, 2010**

(54) **SEPARATION SYSTEMS OF FROZEN-THAWED SPERMATOZOA INTO X-CHROMOSOME BEARING AND Y-CHROMOSOME BEARING POPULATIONS**

(75) **Inventors:** **George E. Seidel, LaPorte, CO (US); Kehuan Lu, Guangxi (CN); Tae Kwang Suh, Fort Collins, CO (US); David G. Cran, Aberdeen (GB)**

(73) **Assignee:** **XY, LLC, Navasota, TX (US)**

(\*) **Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 267 days.

This patent is subject to a terminal disclaimer.

(21) **Appl. No.:** **11/536,576**

(22) **Filed:** **Sep. 28, 2006**

(65) **Prior Publication Data**

US 2007/0042342 A1 Feb. 22, 2007

**Related U.S. Application Data**

(63) Continuation of application No. 10/433,183, filed as application No. PCT/US01/45023 on Nov. 29, 2001.

(60) Provisional application No. 60/253,787, filed on Nov. 29, 2000, provisional application No. 60/253,785, filed on Nov. 29, 2000.

(51) **Int. Cl.**

**A01N 1/02** (2006.01)

**C12Q 1/68** (2006.01)

(52) **U.S. Cl.** ..... 435/2; 435/6

(58) **Field of Classification Search** ..... None  
See application file for complete search history.

(56) **References Cited**

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3,791,517 A 2/1974 Friedman  
3,810,010 A 5/1974 Thorn  
3,816,249 A 6/1974 Bhattacharya  
3,826,364 A 7/1974 Bonner et al.  
3,829,216 A 8/1974 Persidsky

3,833,796 A 9/1974 Fetner et al.  
3,877,430 A 4/1975 Wieder  
3,893,766 A 7/1975 Hogg  
3,894,529 A 7/1975 Shrimpton  
3,906,929 A 9/1975 Augspurger  
3,909,744 A 9/1975 Wisner et al.  
3,944,917 A 3/1976 Hogg et al.  
3,947,093 A 3/1976 Goshima et al.  
3,960,449 A 6/1976 Carleton et al.  
3,963,606 A 6/1976 Hogg  
3,973,003 A 8/1976 Colas  
3,973,196 A 8/1976 Hogg  
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4,014,611 A 3/1977 Simpson et al.  
4,056,324 A 11/1977 Gohde  
4,058,732 A 11/1977 Wieder  
4,067,965 A 1/1978 Bhattacharya  
4,070,617 A 1/1978 Kachel et al.  
4,083,957 A 4/1978 Lang  
4,085,205 A 4/1978 Hancock

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**FOREIGN PATENT DOCUMENTS**

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Weigel, K. A., Exploring the Role of Sexed Semen in Dairy Production Systems; J. Dairy Sci. 87: (E.Supp.): E120-E130; 2004 American Dairy Science Assoc.

(Continued)

**Primary Examiner**—Rebecca E. Prouty

**Assistant Examiner**—Paul C. Martin

(74) **Attorney, Agent, or Firm**—Cindee Ewell; Ryan Christensen

(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 mammal. 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.

**20 Claims, 3 Drawing Sheets**



Acta Número : 20010105573

Resultado de la Búsqueda

|                                                                                 |                                            |                  |              |              |               |
|---------------------------------------------------------------------------------|--------------------------------------------|------------------|--------------|--------------|---------------|
| Fecha Presentación:                                                             | 30/11/2001                                 | Tipo Solicitud : | Patente      | Caracter:    | Independiente |
| Titulo: UN MÉTODO PARA LA TINCIÓN DE CÉLULAS DE ESPERMA RECOLECTADAS DE MAMÍFER |                                            |                  |              |              |               |
| Datos Madre                                                                     | Año                                        | Suc              | Solicitud    |              |               |
| Agente:                                                                         | 908 - 1070 -                               |                  |              |              |               |
| Domicilio Legal:                                                                | VIAMONTE 1145 PISO 10 "A", CAPITAL FEDERAL |                  |              |              |               |
| Resolución Vigente :                                                            | Fec. resolución:                           | 30/03/2010       | Nro:         | AR036869B1   |               |
|                                                                                 | Notificación:                              | 21/04/2010       | Tipo:        | Concedida    |               |
|                                                                                 | SEIDEL, GEORGE E. 0 %                      |                  |              |              |               |
| Titular Vigente:                                                                | SUH, TAE KWANG 0 %                         |                  |              |              |               |
|                                                                                 | LU, KEHUAN 0 %                             |                  |              |              |               |
|                                                                                 | XY, INC. 100 %                             |                  |              |              |               |
| Técnico Titular:                                                                | CARNERO ARIEL                              |                  |              |              |               |
| Publicaciones:                                                                  | Boletín                                    | Fecha            | Publicación  |              |               |
|                                                                                 | 222                                        | 13/10/2004       | AR036869A1   |              |               |
| Prioridades:                                                                    | Número                                     | Fecha            | Páís         |              |               |
|                                                                                 | 60/253785                                  | 29/11/2000       | US           |              |               |
|                                                                                 | 60/253787                                  | 29/11/2000       | US           |              |               |
| Vistas:                                                                         | Descripción                                | Fecha            | Notificación | Contestación |               |
|                                                                                 | E.P.T. 180                                 | 27/06/2003       | 08/09/2003   | 04/06/2004   |               |
|                                                                                 | E.P.T. 180                                 | 10/07/2003       | 08/09/2003   | 04/06/2004   |               |
|                                                                                 | ACLARACION 90                              | 29/12/2008       | 23/01/2009   | 20/04/2009   |               |
|                                                                                 | EXAMEN DE FONDO 60                         | 15/07/2009       | 10/08/2009   | 06/11/2009   |               |
|                                                                                 | I.R.F. 30                                  | 04/01/2010       | 20/01/2010   | 23/02/2010   |               |

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[Ver reivindicaciones](#)

[Ver Resumen](#)

[Ver Escrito](#)

# BOLETIN DE PATENTES



MINISTERIO DE ECONOMIA Y PRODUCCION

SECRETARIA DE INDUSTRIA, COMERCIO Y DE LA PEQUENA Y MEDIANA EMPRESA

INSTITUTO NACIONAL DE LA PROPIEDAD INDUSTRIAL

Nº XXXIX

ISSN - 0325 - 6545

Octubre 13 de 2004 (Edición de 48 páginas)

Nº 222

## CODIGO INID PARA PATENTES Y MODELOS DE UTILIDAD

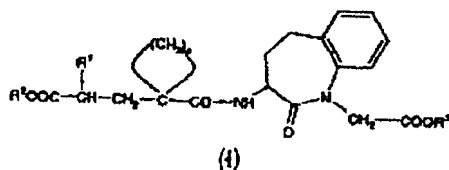
- (10) Identificación del documento
- (21) Número de Solicitud
- (22) Fecha de presentación
- (30) Datos de prioridad
- (41) Fecha de puesta a disposición del público
- (51) Clasif. Internacional de Patentes 6ª o 7ª Edición
- (54) Título de la invención
- (57) Resumen
- (61) Adicional a:
- (62) Divisional de:
- (71) Solicitante:

- (72) Inventor:
- (74) Número Matricula de agente
- (83) Depósito Microorganismos
- Código de tipo doc. según Disposición INPI. Nro. 131/96
- A1= Solicitud de Patente Independiente
- A2= Solicitud de Patente Divisional
- A3= Solicitud de Patente Adicional
- A4= Solicitud de Modelo de Utilidad Independiente
- A5= Solicitud de Modelo de Utilidad Divisional
- A6= Solicitud de Modelo de Utilidad Adicional

## SOLICITUDES DE PATENTE

### PUBLICACIONES DE TRAMITE NORMAL

- ) AR036864 A1
- 1) P000100257
- 2) 20/01/00
- ) DE 199 06 310.9 16/02/99
- 1) A61K 31/55, A61P 9/12
- 1) USO DE COMPUESTOS DE ACIDO BENZACEPIN-N-ACETICO, Y PROCEDIMIENTO PARA LA PREPARACION DE DICHOS COMPUESTOS
- ) Uso de compuestos de la fórmula general (1), en la cual: R<sup>1</sup> representa un radical fenilalquilo inferior eventualmente sustituido en el ciclo fenilo por alquilo inferior, alcoxi inferior o halógeno, o un radical naftil-alquilo inferior; R<sup>2</sup> representa hidrógeno o un radical capaz de formar un éster biolábil; R<sup>3</sup> representa hidrógeno o un radical capaz de formar un éster biolábil; y sales fisiológicamente tolerables de los ácidos de la fórmula (1) para la preparación de composiciones farmacéuticas para el tratamiento de la hipertensión, particularmente de aquellas formas de la hipertensión secundaria causadas por condiciones cardíacas en mamíferos superiores y seres humanos.
- ) SOLVAY PHARMACEUTICALS GMBH  
HANS-BÖCKLER-ALLEE 20, D-30173 HANNOVER, DE
- ) WILKINS, MARTIN R. - WALDECK, HARALD - THORMÄHLEN, DIRK
- ) 190
- ) Fecha: 13/10/2004
- Bol. Nro.: 222



- (10) AR036865 A1
- (21) P000100350
- (22) 27/01/00
- (30) US 09/237405 26/01/99
- (51) C08G 63/08, 63/64, A61K 47/48, 9/16, 9/52, 38/04, C07K 14/432, 7/00
- (54) CONJUGADO MOLECULAR IONICO DE POLIESTERES BIODEGRADABLES Y POLIPEPTIDOS BIOACTIVOS
- (57) Una composición farmacéutica de liberación prolongada. La composición incluye un poliéster que contiene un grupo COOH ionicamente conjugado con un polipéptido bioactivo que comprende al menos una amina ionogénica, donde al menos el 50% por peso del polipéptido presente en la composición es ionicamente conjugado al poliéster.
- Reivindicación 1: Un poliéster que contiene uno o más grupos COOH libres y que tiene una proporción de carboxilo a hidroxilo mayor que uno, donde dicho poliéster contiene un miembro seleccionado del grupo que consiste de ácido láctico-L, ácido láctico-D y ácido láctico DL, ácido málico, ácido cítrico, caprolactona-ε, dioxanona-p, ácido caproico-ε, oxalato de alquilen, oxalato de cicloalquilen, succinato de alquilen, carbonato de trimetileno sustituido y no sustituido, 1,5-dioxepan-2-ona, 1,4-dioxepan-2-ona, glicólido, ácido glicólico, láctido-L, láctido-D, láctido-DL, meso-láctico e isómeros ópticamente activos, racematos o copolímeros de los mismos, siempre que el ácido cítrico, la caprolactona-ε y el glicólido sean miembros del poliéster.
- Reivindicación 5: Una composición que comprende un poliéster, de acuerdo con la Reivindicación 1, ionicamente conjugado a uno o más polipéptidos bioactivos que comprenden al menos una amina iogénica efectiva, donde al menos el 50% por peso del polipéptido presente en la composición es ionicamente conjugado al poliéster.
- Reivindicación 19: Una composición de acuerdo

que durante este lapso, la distancia entre los dos vehículos se extiende considerablemente evitando de esta manera, posibles accidentes o inconvenientes que se dan cuando dos vehículos circulan a corta distancia entre uno y otro.

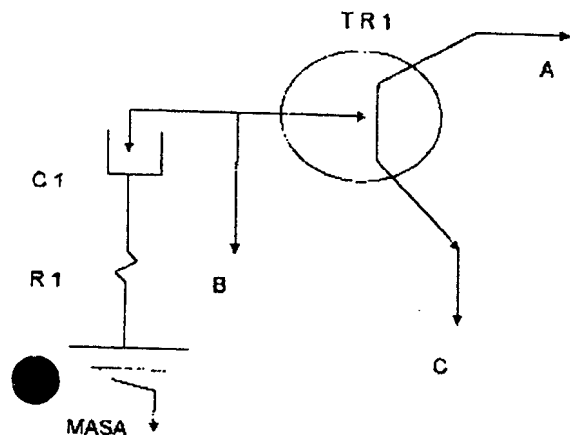
71) MARCOLONGO, IGNACIO JOSE

488 N° 3234 E/ 27 Y 28, CITY BELL, LA PLATA, PROV. DE BUENOS AIRES, AR

72) MARCOLONGO, IGNACIO JOSE

41) Fecha: 13/10/2004

Bol. Nro.: 222



- A) BULBO DE FRENO PATA 1 (12 V)  
B) BULBO DE FRENO PATA 2  
C) CARGA DE LAMPARAS ( CONECTADA ANTES EN PATA 2 DEL BULBO DE FRENO )

0) AR036869 A1

1) P010105573

2) 30/11/01

0) US 60/253785 29/11/00

US 60/253787 29/11/00

1) G01N 33/48, 33/52, 1/30

4) UN METODO PARA LA TINCION DE CELULAS DE ESPERMA RECOLECTADAS DE MAMIFEROS

7) Un método para la tinción de células de esperma recolectadas de mamíferos, cuya finalidad es resolver los problemas que presentan los métodos convencionales en los cuales, por ejemplo, se obtiene una tinción no uniforme, y el tiempo de coloración y la cantidad de colorante adherido al ADN puede afectar la viabilidad de los espermatozoides dando como resultado rangos inferiores de fertilización. El método 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 del 85%.

(71) XY, INC.

1108 NORTH LEMAY AVENUE, FORT COLLINS, COLORADO 80524, US

COLORADO STATE UNIVERSITY (a través de su agente COLORADO STATE UNIVERSITY RESEARCH FOUNDATION)

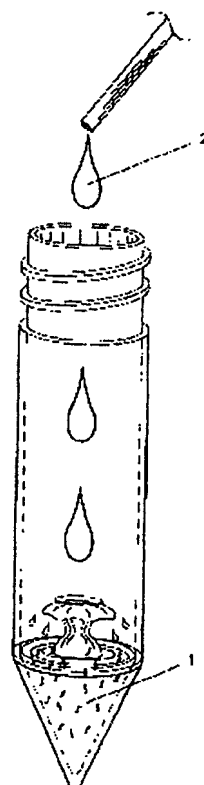
601 SOUTH HOWES STREET, ROOM 410, FORT COLLINS, COLORADO 80549, US

(72) SEIDEL, GEORGE E. - SUH, TAE KWANG - LU, KEHUAN

(74) 908

(41) Fecha: 13/10/2004

Bol. Nro.: 222



(10) AR036870 A1

(21) P010105574

(22) 30/11/01

(30) US 60/253785 29/11/00

US 60/253787 29/11/00

(51) C12N 5/08

(54) METODO PARA TRANSPORTAR OOCITOS

(57) Un método para transportar oocitos, estando este método destinado a evitar la copulación de células espermáticas transportando los oocitos maduros directamente a la instalación donde se separan la células espermáticas tal que estas puedan ser agregadas al medio que contiene oocitos evitando

- (54) Title  
**System to separate frozen-thawed spermatozoa into X-chromosome bearing and Y-chromosome bearing populations**
- (51) International Patent Classification(s)  
**A01N 1/02 (2006.01)**
- (21) Application No: **2002237689** (22) Date of Filing: **2001.11.29**
- (87) WIPO No: **WO02/43574**
- (30) Priority Data
- |                   |                   |              |
|-------------------|-------------------|--------------|
| (31) Number       | (32) Date         | (33) Country |
| <b>60/253,787</b> | <b>2000.11.29</b> | <b>US</b>    |
| <b>60/253,785</b> | <b>2000.11.29</b> | <b>US</b>    |
- (43) Publication Date: **2002.06.11**
- (43) Publication Journal Date: **2002.08.15**
- (44) Accepted Journal Date: **2008.01.10**
- (71) Applicant(s)  
**XY, Inc.;Colorado State University, through its agent, Colorado State University Research Foundation**
- (72) Inventor(s)  
**Lu, Kehuan;Cran, David G;Suh, Tae Kwang;Seidel, George E.**
- (74) Agent / Attorney  
**Freehills Patent & Trade Mark Attorneys, Level 43 101 Collins Street, Melbourne, VIC, 3000**
- (56) Related Art  
**Buchanan et al. Theriogenol. 2000, vol. 53, pp. 1333-1344**  
**Schenk et al. Theriogenology, 1999, vol. 52, pp. 1375-1391.**  
**Seidel et al. Theriogenology, 1999, vol. 52, pp. 1407-1420.**

**(12) STANDARD PATENT APPLICATION**  
**(19) AUSTRALIAN PATENT OFFICE**

12  
214  
(11) Application No. **AU 2008200810 A1**

- (54) Title  
**System to separate frozen-thawed spermatozoa into X-chromosome bearing and Y-chromosome bearing populations**
- (51) International Patent Classification(s)  
**A01N 1/02 (2006.01)**
- (21) Application No: **2008200810** (22) Date of Filing: **2008.02.21**
- (43) Publication Date: **2008.03.13**  
(43) Publication Journal Date: **2008.03.13**
- (62) Divisional of:  
**2002237689**
- (71) Applicant(s)  
**XY, Inc.;Colorado State University**
- (72) Inventor(s)  
**Cran, David G;Suh, Tae Kwang;Seidel, George E;Lu, Kehuan**
- (74) Agent / Attorney  
**Freehills Patent & Trade Mark Attorneys, Level 43 101 Collins Street, Melbourne, VIC, 3000**

**(12) STANDARD PATENT****(11) Application No. AU 2008200810 B2****(19) AUSTRALIAN PATENT OFFICE**

- (54) Title  
**System to separate frozen-thawed spermatozoa into X-chromosome bearing and Y-chromosome bearing populations**
- (51) International Patent Classification(s)  
**C12N 5/00** (2006.01) **A61D 19/00** (2006.01)
- (21) Application No: **2008200810** (22) Date of Filing: **2008.02.21**
- (43) Publication Date: **2008.03.13**  
(43) Publication Journal Date: **2008.03.13**  
(44) Accepted Journal Date: **2010.12.23**
- (62) Divisional of:  
**2002237689**
- (71) Applicant(s)  
**XY, Inc.**
- (72) Inventor(s)  
**Cran, David G;Suh, Tae Kwang;Seidel, George E;Lu, Kehuan**
- (74) Agent / Attorney  
**Freehills Patent & Trade Mark Attorneys, Level 43 101 Collins Street, Melbourne, VIC, 3000**
- (56) Related Art  
**BUCHANAN, B.R. et al., Theriogenology, 2000 (April), vol. 53, pages 1333-1344**

**ANEXO 6.COPIA DE LA RESOLUCIÓN NO. 47353 NOTIFICADA EL 01 DE  
FEBRERO DE 2012.**

**REPÚBLICA DE COLOMBIA  
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO**

**RESOLUCIÓN N° 47 3 5 3**

*Por la cual se deniega una patente de invención*

Rad. PCT/03-054828 A

**EL SUPERINTENDENTE DE INDUSTRIA Y COMERCIO**  
en ejercicio de sus facultades legales, en especial de las conferidas en el numeral  
38 del artículo 3° del Decreto 1687 de 2010 y

**CONSIDERANDO:**

**PRIMERO:** Que mediante escrito radicado en esta Superintendencia el 27 de junio de 2003, con el número 03-054828, por el doctor Emilio Ferrero, en su calidad de apoderado de XY, INC. y COLORADO STATE UNIVERSITY, por medio de su agente, COLORADO STATE UNIVERSITY RESEARCH FOUNDATION, entró a fase nacional la solicitud de patente de invención titulada "DISPOSICIÓN PARA SEPARAR ESPERMATOZOIDES CONGELADOS EN POBLACIONES QUE CONTIENEN CROMOSOMAS X y CROMOSOMAS Y", que corresponde a la solicitud internacional PCT/US01/45023 del 29 de noviembre de 2001.

**SEGUNDO:** Que efectuado el examen de forma, en cuanto a los requisitos que debe reunir la solicitud y cumplidas las exigencias legales establecidas en la Decisión 486 de la Comisión de la Comunidad Andina, el Tratado de Cooperación en materia de Patentes (PCT), el reglamento y la Circular Única de la Superintendencia de Industria y Comercio, se ordenó publicar el extracto sin que se hubieran presentado oposiciones por parte de terceros.

**TERCERO:** Que la solicitante XY, INC., mediante escrito radicado el 19 de enero de 2009, informó sobre la cesión de derechos, respecto de la presente solicitud de patente, de XY, INC. y COLORADO STATE UNIVERSITY, por medio de su agente, COLORADO STATE UNIVERSITY RESEARCH FOUNDATION a XY, INC., la cual se encontró que reúne los requisitos legales.

**CUARTO:** Que mediante escrito radicado el 23 de febrero de 2010, la solicitante allegó la presente solicitud divisional No. 03 – 054828 A, la cual se derivó de la mencionada solicitud inicial 03 – 054828 y, por ende, se beneficia de la fecha de su presentación. Adicionalmente, se allegó sustitución de poder de Emilio Ferrero a favor de Luz Clemencia de Páez, la cual se encontró que reúne los requisitos legales.

**QUINTO:** Que realizado el examen de patentabilidad de la presente solicitud divisional, se encontró que la invención no es patentable. En consecuencia, con fundamento en lo dispuesto en el artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se requirió a la solicitante mediante Oficio No.6424,

REPÚBLICA DE COLOMBIA  
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RESOLUCIÓN N° 47353  
*Por la cual se deniega una patente de invención*

Rad. PCT/03-054828 A

notificado el 14 de mayo de 2010 y Oficio No. 14756, notificado el 12 de noviembre del mismo año, para que se pronunciara sobre las objeciones efectuadas a la solicitud, en cuanto a la carencia de nivel inventivo, de acuerdo a las exigencias del artículo 18 de la Decisión 486 mencionada.

**SEXTO:** Que la solicitante, mediante escritos radicados el 21 de septiembre de 2010 y el 4 de febrero de 2011, atendió oportunamente los requerimientos formulados y presentó aclaraciones. Con cada una de las respuestas se allegó un nuevo capítulo reivindicatorio. El primero de ellos, se ajusta a las prescripciones del artículo 34 de la Decisión 486 y el segundo no se ajusta a dichas prescripciones, razón por la cual la decisión de la presente Resolución se toma con base en el capítulo reivindicatorio allegado con la respuesta del 21 de septiembre de 2010.

**SEPTIMO:** Que la solicitante XY, INC., mediante escrito radicado el 24 de noviembre de 2010, informó el cambio de nombre de XY, INC. a XY, LLC, el cual se encontró que reúne los requisitos legales.

**OCTAVO:** Que según lo dispuesto en el artículo 14 de la Decisión 486 expedida por la Comisión de la Comunidad Andina se otorgarán patentes "(...) para las invenciones, sean de producto o de procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial".

**NOVENO:** Que las reivindicaciones 1 a 9, contenidas en los folios 142 a 144, carecen de nivel inventivo. Lo anterior, de acuerdo con las siguientes consideraciones:

#### 9.1. Objeto de la invención

El objeto de la invención se refiere a un método para diferenciar espermatozoides y permitir su separación en poblaciones que contienen cromosomas X y cromosomas Y.

##### 9.1.1. Problema técnico que resuelve la invención

El problema técnico que resuelve la invención consiste en la necesidad de separar espermatozoides que contienen cromosomas X de espermatozoides que contienen cromosomas Y, de manera tal que cada población resultante contenga un número significativo de espermatozoides separados de forma correcta y uniforme.

**REPÚBLICA DE COLOMBIA  
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO**

**RESOLUCIÓN N° 47353**  
*Por la cual se deniega una patente de invención*

Rad. PCT/03-054828 A

**9.1.2. Solución al problema técnico**

Para resolver el problema técnico, la solicitud en estudio proporciona una disposición de citómetro de flujo para aislar células de esperma deseadas.

**9.2. Modificaciones no aceptadas**

El artículo 34 de la Decisión 486 establece que: *"El solicitante de una patente podrá pedir que se modifique la solicitud en cualquier momento del trámite. La modificación no podrá implicar una ampliación de la protección que correspondería a la divulgación contenida en la solicitud inicial"*

Al respecto, el Tribunal de Justicia de la Comunidad Andina señala lo siguiente:

*"La única limitación impuesta al solicitante respecto de la modificación consiste en que ésta no debe implicar una **ampliación** del invento o de la divulgación contenida en la solicitud presentada. Contrario sensu, la modificación de la solicitud podrá concretar el invento o reducir el alcance de las reivindicaciones, sin importar cuál haya sido la redacción originariamente propuesta; por lo que aun cuando el cambio sea sustancial con relación a las reivindicaciones iniciales, lo único relevante viene a ser que las nuevas reivindicaciones puedan seguir siendo sustentadas racionalmente con fundamento en la descripción contenida en la solicitud inicial. [Proceso 25-IP-2002, sentencia de 8 de mayo del 2002. Publicado en Gaceta Oficial N°769, de 24 de mayo del 2002. Patente: 'IMINODERIVADOS CICLICOS'. TRIBUNAL DE JUSTICIA DE LA COMUNIDAD ANDINA]" -negrilla original-<sup>1</sup>*

En el presente caso, las reivindicaciones 1 a 20 (Fls. 181 a 185) allegadas con la respuesta al Oficio No. 14756 de 2010, radicada el 4 de febrero de 2011, no son aceptadas, porque mencionan un método para teñir células de esperma, con diferentes características técnicas, que no había sido revelado inicialmente, razón por la cual las reivindicaciones amplían el objeto de la solicitud.

Es preciso aclarar, que la solicitud divisional que ahora se estudia se refería a una *"disposición de citómetro de flujo para aislar células de esperma"* (capítulo reivindicatorio con 9 reivindicaciones, Fls. 99 a 101) y, posteriormente, fue modificada por un *"método para aislar células de esperma"* (capítulo reivindicatorio modificado con 9 reivindicaciones, Fls. 142 a 144), capítulos que guardan el objeto de la invención contenido en la divisional, con sus características comunes, sin incluir materia adicional.

<sup>1</sup> PROCESO 49-IP-2010. TRIBUNAL DE JUSTICIA DE LA COMUNIDAD ANDINA. Junio 23 de 2010.  
Página 3 de 10

REPÚBLICA DE COLOMBIA  
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RESOLUCIÓN N° 47353

*Por la cual se deniega una patente de invención*

Rad. PCT/03-054828 A

El capítulo reivindicatorio fue modificado nuevamente por la solicitante, mediante la presentación del mismo capítulo de la solicitud originalmente radicada con el número 03 – 54828 (solicitud inicial de la cual se derivó la presente divisional). El objeto de este último capítulo consiste en un "método para teñir células de esperma", el cual, aparte de ser materia ya analizada y negada, incluye reivindicaciones que en sus características técnicas no eran parte de la presente divisional, tal como fue aceptada inicialmente.

**9.3. Determinación del estado de la técnica**

**9.3.1. Fecha determinante para el análisis comparativo**

La fecha para determinar el estado de la técnica es el 29 de noviembre de 2000, que corresponde a la fecha de las prioridades invocadas respecto de las solicitudes US Nos. 60/253,785 y 60/253,787.

La fecha de la prioridad fue tomada en cuenta para el estudio de patentabilidad, según obra a folio 189 del expediente en estudio.

**9.3.2. Documentos del estado de la técnica**

Consultadas las fuentes de información con que se cuenta, se encontró el documento US 6.149.867, titulado "Sheath fluids and collection systems for sex-specific cytometer sorting of sperm", con fecha de publicación del 21 de noviembre de 2000, anterior a la fecha de las prioridades invocadas y relacionado con la materia de la solicitud en estudio.

**9.3.3. Información derivada del antecedente**

La anterioridad US 6.149.867 (de aquí en adelante denominada D1), divulga un sistema mejorado de citómetro de flujo, adaptado para ser usado en la selección de esperma por diferenciación del sexo, que incluye hacer pasar las células a través de un fluido envainado para disminuir las fuerzas en dicho fluido. La invención describe, además, el proceso de teñido del semen para ser detectado por un sistema de láser, en el cual las longitudes de onda de los cromosomas Y y X, al ser teñidos, diferencialmente permiten la separación de cada muestra de semen.

**9.4. Nivel inventivo**

Según lo dispuesto en el artículo 18 de la misma Decisión 486 "se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente

**REPÚBLICA DE COLOMBIA**  
**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO**

**RESOLUCIÓN N° 47 353**

**Por la cual se deniega una patente de invención**

Rad. PCT/03-054828 A

*versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica".*

El estado de la técnica, al tenor de lo dispuesto en el artículo 16 de la misma Decisión, comprende "todo lo que haya sido accesible al público, por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso, de la prioridad reconocida".

**El Tribunal de Justicia de la Comunidad Andina ha dicho sobre este requisito lo siguiente:**

*“Como segundo requisito, adicional al de la novedad, se exige que la invención tenga nivel inventivo, esto es, que implique un salto cualitativo en relación con la técnica existente. Este requerimiento es desarrollado por el artículo 4 de la Decisión 344 que presupone el requisito de nivel inventivo al establecer que la invención goza de dicho nivel al no derivar de manera evidente del estado de la técnica, ni resultar obvia para una persona entendida o versada en la materia.*

*Este requisito ofrece al examinador la posibilidad de determinar si con los conocimientos técnicos que existían al momento de la invención, se hubiese podido llegar de manera evidente a la misma, o si el resultado hubiese sido obvio para un experto medio en la materia de que se trate, es decir, para una persona del oficio y normalmente versada en la materia técnica correspondiente.*

**Con respecto a lo anterior, el Tribunal de Justicia de la Comunidad Andina ha manifestado lo siguiente:**

*'Con el requisito del nivel inventivo, lo que se pretende es dotar al examinador técnico de un elemento que le permita afirmar o no si a la invención objeto de estudio no se habría podido llegar a partir de los conocimientos técnicos que existían en ese momento dentro del estado de la técnica... En este punto conviene advertir que uno es el examen que realiza el técnico medio respecto de la novedad y otro el que se efectúa con respecto al nivel inventivo; si bien en uno y otro se utiliza como parámetro de referencia el 'estado de la técnica', en el primero, se coteja la invención con las 'anterioridades' existentes dentro de aquella, cada uno (sic) por separado, mientras que en el segundo (nivel inventivo) se exige que el técnico medio que realiza el examen debe partir del conocimiento general que él tiene sobre el estado de la técnica y realizar el cotejo comparativo con su apreciación de conjunto, determinando si con tales conocimientos técnicos existentes ha podido o no producirse tal invención'*

**REPÚBLICA DE COLOMBIA  
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO**

**RESOLUCIÓN N° 47353**

*Por la cual se deniega una patente de invención*

Rad. PCT/03-054828 A

[Proceso N° 12-IP-98. Doc. Cit. TRIBUNAL DE JUSTICIA DE LA COMUNIDAD ANDINA].

*Resulta pertinente, a los efectos del artículo 4 de la Decisión 344, definir qué se entiende por 'obvio' y por 'evidente' a fin de determinar qué se debe entender por nivel inventivo. Lo obvio es aquello que salta a la vista de manera clara y directa; lo 'que se encuentra o pone delante de los ojos, muy claro o que no tiene dificultad'. Lo evidente consiste en la 'certeza clara y manifiesta de la que no se puede dudar, cierto, claro, patente y sin la menor duda' (DICCIONARIO DE LA LENGUA ESPAÑOLA. Vigésima Segunda Edición. ESPASA. Madrid, España. 2001. 'DEFINICIONES DE EVIDENTE, EVIDENCIA Y OBVIO'. Tomos 5 y 7. Págs. 686 y 1089).*

*En este orden de ideas, se puede concluir que una invención goza de nivel inventivo cuando a los ojos de un experto medio en el asunto de que se trate, se necesita algo más que la simple aplicación de los conocimientos técnicos en la materia para llegar a ella, es decir, que de conformidad con el estado de la técnica el invento no es consecuencia clara y directa de dicho estado".<sup>2</sup>*

Visto lo anterior, se advierte que el método para diferenciar espermatozoides, reivindicado en la presente solicitud carece de nivel inventivo, porque a los ojos de una persona del oficio normalmente versada en la materia técnica se deriva, de manera evidente, a partir de la información enseñada por el estado del arte.

En efecto, la reivindicación 1 divulga un método para aislar células de espermatozoides que comprende las etapas de:

| Características esenciales                                                                                                                       | D1                                                               |
|--------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------|
| Obtener células de espermatozoides mediante descongelar semen previamente congelado.                                                             | - (Se menciona con fines de transporte, ejemplos 1 a 3)          |
| Incubar las células de espermatozoides con colorante fluorocromo en una concentración micro-molar de entre 40 y 2500 por entre 50 y 200 minutos. | - (Se ejemplifica con 38µm de colorante) (Col. 15, líneas 2 a 8) |
| Suministrar dichas células de espermatozoides a un citómetro de flujo.                                                                           | √ (Col. 5, línea 47)                                             |
| Crear un entorno de fluido de envainado dentro de dicho citómetro de flujo en el cual dichas células de espermatozoides están retenidas.         | √ (Col. 5, 3° y 4° párrafo)                                      |
| Pasar dichas células de espermatozoides a través de una boquilla mientras están retenidas en dicho entorno de fluido envainado                   | √ (Col. 5, 4° párrafo)                                           |

<sup>2</sup> PROCESO 95-IP-2010. TRIBUNAL DE JUSTICIA DE LA COMUNIDAD ANDINA. Noviembre 11 de 2010. Publicada el 27 de enero de 2011.

**REPÚBLICA DE COLOMBIA**  
**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO**

**RESOLUCIÓN Nº 47353**  
*Por la cual se deniega una patente de invención*

Rad. PCT/03-054828 A

|                                                                                                                                                                                                            |                            |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------|
| pasando a través de un sistema de detección.                                                                                                                                                               |                            |
| Excitar dichas células de esperma con una fuente de radiación.                                                                                                                                             | √ (Col. 6, líneas 12 y 13) |
| Detectar la magnitud de fluorescencia emitida por el enlace fluorocromo al ADN del esperma con una disposición sensora de células de esperma que responde a dichas células de esperma.                     | √ (Col. 6, líneas 16 a 19) |
| Separar dichas células de esperma que tienen una característica deseada, basándose en la magnitud de fluorescencia emitida por cada una de las células con una disposición de separación y discriminación. | √ (Col. 6, 2º párrafo)     |

El documento del estado de la técnica más cercano es D1 (Fls. 103 a 111), que enseña la forma de separar cromosomas X de cromosomas Y, mediante un citómetro de flujo, tal como se observa en la tabla. Adicionalmente, con respecto a la solicitud, divulga la misma disposición del citómetro mediante una fuente de células de esperma, fuente de fluido envainado, la boquilla a través de la cual pasan dichas células de esperma, el oscilador que actúa en dicho fluido envainado, el elemento sensor de las células de esperma, una disposición que discrimina las células según sus características de tinción (con incluso el mismo principio de tinte reivindicado por la solicitud) y el elemento de contención para coleccionar dichas células de esperma. En el ejemplo 1 enseñado en dicha anterioridad, se señala la forma de tinción de células de esperma mediante su incubación a 38 µm de colorante Hoechst 3342 por una hora a 34°C. De esta forma, se alcanzó una pureza de 90%, aproximadamente (Col. 15, línea 9 y Col. 16, línea 33). Los experimentos llevados a cabo en D1 involucran el congelamiento y descongelamiento del semen con fines de su transporte. Dicho mecanismo, por lo tanto, es común en el estado de la técnica, antes o después de la tinción.

Las diferencias de la solicitud frente a D1, radican en la mayor concentración de colorante (40 – 20500 micro-molar). Estas diferencias, relacionadas con las condiciones a las cuales se realiza el proceso de tinte y de mantenimiento de las células no demuestran un salto sorprendente en la separación de cromosomas, ya que en D1 se logró una eficiencia en la separación del 90% (Col. 15, líneas 8 a 10), mientras que en la solicitud la eficiencia para separar poblaciones de cromosomas es de más del 85%.

Por otra parte, puesto que D1 divulgaba previamente la necesidad de separar espermatozoides que contienen cromosomas X de espermatozoides que contienen cromosomas Y e indicaban que cada población resultante tuviera un número significativo de espermatozoides separados de forma correcta e uniforme, una persona del oficio normalmente versada en la materia, motivada por tal

**REPÚBLICA DE COLOMBIA  
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO**

**RESOLUCIÓN N° 47 3 5 3**

*Por la cual se deniega una patente de invención*

Rad. PCT/03-054828 A

sugerencia, lograría métodos presentados en la solicitud basándose en las enseñanzas divulgadas en D1.

En consecuencia, el método reivindicado carece de nivel inventivo, ya que se deriva de manera evidente del estado del arte y resulta obvio para una persona normalmente versada en la materia técnica y, por ende, no cumple con lo dispuesto en el artículo 18 de la Decisión 486 de la Comisión de la Comunidad Andina.

**9.4. Sobre la respuesta de la solicitante**

Con relación a los argumentos de la solicitante, allegados con motivo de la notificación de fondo, se determina que:

La solicitante argumenta que *"aunque (como lo afirma el examinador) D1 da a conocer (a través de los ejemplos 1, 2 y 3) el congelamiento y el descongelamiento de células de esperma (con el transporte asociado), D1 no divulga, enseña o sugiere el congelamiento y el descongelamiento de células de esperma con la subsecuente tinción del mismo o el proceso de tinción seguido por el congelamiento y descongelamiento del esperma"* (Fl. 174).

Al respecto, tal como se ha determinado en el análisis de patentabilidad, las diferencias de la solicitud y la anterioridad radican en la separación de esperma, previamente congelado y descongelado, o de semen fresco.

Debido a que no se evidencia un efecto técnico sorprendente, y el estado de la técnica indica el separar, mediante una metodología similar a la reivindicada, esperma de variada procedencia, la solicitud continúa careciendo de nivel inventivo. En este orden de ideas, cualquier esperma de mamífero que presenta las diferencias estructurales evidentes en los cromosomas X y Y, es susceptible a separación por la metodología divulgada en D1, ya que la solicitud presenta el mismo fundamento técnico.

La característica técnica de congelar y descongelar el semen antes del tratamiento, no evidencia efectos técnicos sorprendentes ligados a diferencias estructurales con fines de separación del esperma, como se demostró en el análisis de patentabilidad. De esta manera, los argumentos de la solicitante no son procedentes.

La solicitante menciona que *"debido a que D1 no revela el proceso de separación de esperma teñido que había sido previamente congelado y posteriormente descongelado, una persona versada en la materia haría caso omiso en cuanto a*

Página 8 de 10

Sede Centro: Carrera 13 No. 27-00 Pisos 2, 5, 7 y 10  
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tabica colocada para el análisis  
tenida a la vista

REPÚBLICA DE COLOMBIA  
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RESOLUCIÓN N° 47353

*Por la cual se deniega una patente de invención*

Rad. PCT/03-054828 A

*que el espermatozoide congelado y posteriormente descongelado pudiera ser separado"*  
(Fl. 175).

El anterior argumento es cierto, en la medida en que la solicitud prueba efectivamente que es posible separar semen previamente congelado y descongelado, pero el procedimiento para realizarlo es el mismo que el divulgado por el documento del estado de la técnica (D1). Según este argumento, dicha característica resultaría ser un objeto a lograr del método y no un paso necesario para la efectiva separación del material cromosómico, cuya separación da el mismo resultado y no una ventaja técnica para el producto final.

Asegura, que *"con el fin de teñir de manera uniforme la muestra más densa de espermatozoides descongelados, uno tendría que teñir la muestra durante más tiempo o con una mayor concentración del tinte. Sin embargo, una persona versada en la materia también conoce que el proceso de tinción es perjudicial para los espermatozoides, y el aumento del tiempo de tinción o el aumento de la concentración de colorante tendría un impacto negativo sobre las características de fertilidad de los espermatozoides"*.

Ante este argumento, es preciso hacer énfasis en que los valores empleados de tinción de concentración de colorante, tiempo y temperatura son muy similares, siendo, de hecho, menores en la anterioridad, demostrándose buena cantidad (90%) en la viabilidad. Por tanto, el problema que se plantea en este argumento ya estaba resuelto en D1, tal como se observó en el análisis de patentabilidad.

Asegura, que *"el hecho que el Examinador afirme que el mecanismo de congelamiento y descongelamiento de espermatozoides revelado en la presente solicitud es normal en el estado de la técnica antes o después de la tinción corresponde a una simple especulación y a un análisis ex post facto de la solicitud"* (Fl. 175).

Al respecto, el análisis de nivel inventivo no fue realizado en forma retrospectiva, toda vez que el examen de patentabilidad se realizó con base en los conocimientos que tenía el técnico de nivel medio en la materia para la época de presentación de la solicitud, así como con el conocimiento divulgado en el documento previamente publicado. Por lo tanto, a partir de estos conocimientos era perfectamente posible lograr el objeto reivindicado en la presente invención.

En conclusión, teniendo en cuenta que los argumentos de la solicitante no desvirtúan de manera alguna las objeciones a la patentabilidad ya sustentadas, el Superintendente de Industria y Comercio,

SUPERINTENDENCIA

REPÚBLICA DE COLOMBIA  
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RESOLUCIÓN N° 47353

Por la cual se deniega una patente de invención

Rad. PCT/03-054828 A

## RESUELVE:

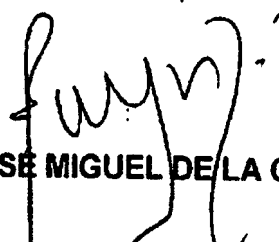
**ARTÍCULO PRIMERO:** Denegar la patente de invención a la creación titulada "DISPOSICIÓN PARA SEPARAR ESPERMATOZOIDES CONGELADOS EN POBLACIONES QUE CONTIENEN CROMOSOMAS X y CROMOSOMAS Y".

**ARTÍCULO SEGUNDO:** Notificar personalmente el contenido de la presente resolución a la doctora Luz Clemencia de Páez, en su calidad de apoderada sustituta de la sociedad XY, LLC, o a quien haga sus veces, entregándole copia de la misma y advirtiéndole que contra ella procede el recurso de reposición, ante el Superintendente de Industria y Comercio, el cual podrá ser interpuesto en el momento de la notificación o dentro de los 5 días hábiles siguientes a ella.

## NOTIFÍQUESE Y CÚMPLASE

Dada en Bogotá D.C., a los 31 AGO. 2011

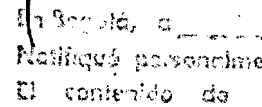
EL SUPERINTENDENTE DE INDUSTRIA Y COMERCIO,

  
 JOSÉ MIGUEL DE LA CALLE RESTREPO

01 FEB. 2012

Doctora  
LUZ CLEMENCIA DE PAEZ  
Carrera 4ª No. 72 - 35.  
Bogotá D.C.

Elaboró: Laura Victoria Camacho C.   
Revisó: Doris Elena Polo Cal   
Revisó: José Luis Salazar L.   
Aprobó: José Luis Londoño F. 

En Bogotá, a   
Notifiqué personalmente a Dr. LEON MARCELO REYES  
El contenido de la anterior providencia que  
impuesto firma

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Página 10 de 10

Sede Centro: Carrera 13 No. 27-60 Pisos 5, 7 y 10  
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Bogotá D.C. - Colombia

NOTIFICADOR  
Hace constancia de la notificación de la presente resolución a la doctora Luz Clemencia de Páez, en su calidad de apoderada sustituta de la sociedad XY, LLC, o a quien haga sus veces, entregándole copia de la misma y advirtiéndole que contra ella procede el recurso de reposición, ante el Superintendente de Industria y Comercio, el cual podrá ser interpuesto en el momento de la notificación o dentro de los 5 días hábiles siguientes a ella.

## TABLA DE CONTENIDO

| Documento                                                                                                                                                                                                             | Página |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------|
| Recurso de reposición contra la Resolución 47353 de Agosto 31 de 2011.....                                                                                                                                            | 1      |
| Anexo 1. Nuevo capítulo reivindicatorio modificado, formulario de<br>modificación y pago de la tasa por modificación.....                                                                                             | 19     |
| Anexo 2. Declaración Dr. Rick Lenz.....                                                                                                                                                                               | 25     |
| Anexo 3. Artículo “Storage of bovine semen in liquid and frozen state”<br>R. Vishwanath y P. Shannon.....                                                                                                             | 31     |
| Anexo 4. Publicación de patente WO2004/012837.....                                                                                                                                                                    | 49     |
| Anexo 5. Copia de la primera página de la publicación de patentes en<br>paralelas para Estados Unidos US 7,713,687 y US 7,771,921, Argentina<br>AR 036869B1 y Australia 2002237689, 2008200810 A1 y 2008200810B2..... | 68     |
| Anexo 6.copia de la resolución no. 47353 notificada el 01 de febrero de 2012...                                                                                                                                       | 73     |