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Referencia : Expediente No. 03.054.828  
Asunto : Solicitud de patente para DISPOSICIÓN DE  
SEPARAR ESPERMATOZOIDES CONGELADOS EN  
POBLACIONES QUE CONTIENEN CROMOSOMAS X y  
CROMOSOMAS Y.  
Solicitante : XY, INC.  
Clase : A01K 067/000, A01K 067/033, A61B, C12N 005/000  
Fecha de Solicitud: 27 de junio de 2003

1. Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, me refiero al oficio No. 6423 notificado por fijación en lista del 14 de mayo de 2010.

2. Mediante memorial radicado el día 6 de agosto de 2010 solicité plazo de treinta (30) días para atender el oficio No. 6423.

3. Mediante oficio No. 6423 se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente. Las observaciones realizadas en el oficio son las siguientes:

**3.1 Análisis de patentabilidad****3.1.1 Nivel Inventivo**

Se objeta el nivel inventivo de la presente solicitud, en vista de las enseñanzas del documento D1: US 6149867.

De acuerdo con los argumentos del Examinador, la publicación del estado del arte D1 enseña una forma de separar cromosomas X de cromosomas Y mediante un clitómetro de flujo, en donde se revelan experimentos que involucran el congelamiento y descongelamiento del semen con fines de su transporte.

De esta manera, afirma el Examinador que la diferencia entre la presente solicitud y D1 radica en que la concentración empleada en D1 es menor, pero muy cercana al límite inferior del intervalo reclamado en la presente solicitud (40-2500µm); y que debido a que dicha diferencia no evidencia un efecto sorprendente, las reivindicaciones no cumplen con el requisito de nivel inventivo.

#### **4. Respuesta a las objeciones**

De este modo y con el objeto de atender las objeciones arriba mencionadas, se desean poner de presente las siguientes aclaraciones y argumentos:

##### **4.3 Argumentos a favor del nivel inventivo de la solicitud**

En concordancia con el artículo 18 de la Decisión 486, se establece que una solicitud es inventiva cuando la solución técnica no resulta obvia a partir de las enseñanzas contenidas en el arte previo.

El Examinador ha citado un documento el cual considera afecta el nivel inventivo de la presente solicitud, es decir, que una persona medianamente versada en la materia podría derivar de manera obvia la solución técnica aquí reivindicada, en vista de enseñanzas o sugerencias contenidas en dicho documento.

De otro lado, el Examinador no considera que las ventajas contribuidas por la presente invención y relacionadas con la obtención de una disposición para separar espermatozoides congelados a alta pureza y manteniendo la viabilidad de la esperma.

En vista de lo anterior y de manera respetuosa manifestamos que nos apartamos de la opinión del Examinador cuando afirma que la presente invención no es inventiva a la luz de las enseñanzas de D1. Con respecto a lo anterior deseamos poner de presente los siguientes

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argumentos que demuestran que la anterioridad citada no guía a una persona versada en la materia a obtener de manera obvia la presente solicitud:

Inicialmente, deseamos llamar la atención del Examinador en cuanto que la anterioridad D1 enseña una forma de separar cromosomas X de cromosomas Y; sin embargo, dicha anterioridad se refiere a la separación de semen fresco y en ningún momento hace referencia a la separación de semen previamente congelado. En consecuencia, a pesar de la afirmación formulada en la Acción Oficial anterior, no hay ningún indicio o sugerencia en D1 para separar esperma descongelado que había sido previamente congelado.

De hecho, 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. La yema de huevo puede presentar problemas en el flujo típico de separación; a este respecto la anterioridad D1 enseña el hecho de añadir la yema de huevo después de la tinción (Ver Columna 13, líneas 24 a 26). 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.

Adicionalmente, consideramos importante aclarar 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. De esta manera, una de las reivindicaciones de la anterioridad corresponde a la creación de una

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muestra de inseminación con dosis baja debido a que el esperma pierde motilidad a lo largo del proceso de separación debido a que en el tiempo que tomaría separar el esperma suficiente para una dosis convencional la mayoría de los espermatozoides perderían su motilidad progresiva, y sus características de fertilidad serían muy pobres. Por consiguiente, debido a esta pérdida de la motilidad por el proceso de tinción por sí solo, la persona versada en la materia creía anteriormente que el esperma debilitado por el congelamiento no produciría muestras de esperma viables, tal como se obtiene en la presente solicitud.

De otro lado, deseamos poner de presente que tal como se demuestra a continuación, ninguno de los ejemplos de D1 provee la información necesaria para que una persona versada en la materia se viera motivada a derivar de manera obvia la presente solicitud a partir de las enseñanzas de dicha anterioridad. → ?

El ejemplo 1 de la anterioridad D1 describe el proceso de separación de "semen recién extraído", el cual fue enfriado de 3 a 5°C y usado para inseminación 5 a 9 horas después de ser separado. Lo anterior, es muestra de una gran diferencia con respecto a lo revelado en la presente solicitud, que se refiere a "esperma congelado-descongelado".

✓ Por esta razón, el método de la presente solicitud de patente proporciona una solución, la cual es muestra del nivel inventivo involucrado y que permite la separación de esperma congelado-descongelado, lo que no solo no había sido logrado anteriormente, sino que la persona versada en la materia consideraba que no era posible lograrlo. ?

A este respecto, deseamos allegar una copia de la solicitud US 60/253,787 correspondiente a la prioridad de la presente solicitud. La tabla 1 de dicho documento muestra que el esperma congelado-descongelado genera blastocitos a aproximadamente 70% de niveles de éxito comparable a los resultados de esperma sin congelar o esperma sin separar. Lo anterior, es muestra de la ventaja en la viabilidad del esperma obtenida por medio de la presente solicitud.

En el ejemplo 2 de la anterioridad D1 se describe la recolecta de esperma y el congelamiento del mismo; sin embargo, no menciona el proceso de separación del esperma. El propósito de dicho experimento

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era determinar la tasa de embarazo cuando se inseminaban novillas con bajos números de esperma en condiciones ideales.

En el ejemplo 3 el esperma fue recolectado, separado y usado para inseminar entre 9 y 29 horas después del proceso de separación. Dicho esperma fue recolectado como fresco y dicho ejemplo no revela o sugiere que dicho esperma era congelado y después descongelado antes de la separación.

Finalmente, el ejemplo 4 revela inseminación con bajo número de esperma en donde el esperma ni ha sido congelado ni separado.

Por consiguiente, al tomar las enseñanzas de dichos ejemplos solos o en combinación, no es posible afirmar que la materia revelada en la presente solicitud se encuentre sugerida en el arte previo. 2

Adicionalmente, deseamos reiterar que para el caso en particular de la separación de esperma bovino, se debe tener en cuenta que para el esperma bovino hay aproximadamente una diferencia del 3,8% entre la cantidad de ADN presente en una célula de esperma que contiene cromosomas X y la que contiene cromosomas Y. Esto se refleja en la cantidad de fluorescencia emitida por cada una de las células de esperma teñidas al ser excitadas por el láser. Con el fin de lograr la suficiente resolución en cada una de las fluorescencias de las células de esperma, cada célula en la muestra debe estar uniformemente teñida. Es por esto que sin una tinción suficientemente uniforme de las células no sería posible realizar una separación de alta pureza, como la reivindicada en la presente invención, debido a que existiría un solapamiento grande entre magnitudes de fluorescencia. Este solapamiento no se alcanzaría a compensar con la diferencia del 3.8% de la cantidad de ADN existente entre los espermatozoides que contienen cromosomas X y los que contienen Y. 2

De esta manera, una persona versada en la materia es consiente que las células de esperma son delicadas y que elevadas concentraciones de Hoechst 33342 tienen un impacto negativo en la viabilidad del las 3

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células de esperma. Igualmente, los tiempos elevados de tinción tienen un impacto negativo en la viabilidad de las células de esperma.

A este respecto, deseamos poner de presente que la Tabla 1 de la presente solicitud muestra la contribución de dicha 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 esperma mientras se mantiene la viabilidad de las mismas. Dicha tabla provee la información necesaria relacionada al desarrollo embrionario, lo que es muestra de que las concentraciones de la tintura y el tiempo de tinción puede producir células de esperma separadas viables.

En consecuencia, la reivindicación 1 provee una solución real a un problema técnico más allá de lo enseñado o sugerido en el estado del arte y provee un efecto técnico inesperado debido a que específicamente, no existe enseñanza o sugerencia para los tiempos de tinción y concentraciones de tintura que la presente solicitud ha encontrado para teñir células de esperma congeladas-descongeladas con la uniformidad necesaria para que sean separadas a alta pureza y manteniendo la viabilidad de las mismas.

Solicitamos atentamente tener en cuenta los argumentos anteriores, los cuales consideramos demuestran de manera suficiente que la presente solicitud es tanto novedosa como inventiva sobre el arte previo de manera que las objeciones formuladas a este respecto se ven superadas.

Puesto que se han atendido a cabalidad las observaciones hechas por su Despacho sobre la presente solicitud de patente, respetuosamente solicito se continúe con su tramitación. Sin embargo, en el evento que el Despacho considere que una modificación adicional es necesaria, respetuosamente le solicito hacer uso de la facultad que le otorga el artículo 45 inciso 2 de la Decisión 486 de la Comisión de la Comunidad Andina a efecto de requerir nuevamente de nuestra parte cualquier complementación o aclaración.

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5. ANEXOS

- Copia de la solicitud US 60/253,787

Señora Directora,

*Luz Clemencia de Páez*  
LUZ CLEMENCIA DE PÁEZ

C.C. 35.456.344 de Usaquén

T.P.A. No. 23.555

COLO-13534-843-002-0

COLO-13842-841-001-1

DVF\DVF\ID-178818\WPC13534

No. M-2010-178818\DVF

## A SYSTEM FOR IN-VITRO FERTILIZATION WITH SPERMATOZOA SEPARATED INTO X-CHROMOSOME AND Y-CHROMOSOME BEARING POPULATIONS

### BACKGROUND OF THE INVENTION

Devices, compositions, and methods that promote early cleavage and improve the quality of embryos generated using in-vitro fertilization (IVF) with spermatozoa separated into X-chromosome bearing and Y-chromosome bearing populations.

An attractive feature of IVF is that many fewer spermatozoa can be required for insemination than for artificial insemination. However, IVF using spermatozoa separated into X-chromosome bearing and Y-chromosome bearing populations (separated spermatozoa) can necessitate modifications to conventional IVF techniques. This may be due in part to the pre-capacitation of such spermatozoa.

In most cases, the percentages of oocytes (oocyte, ootid, or ova, or plurality of same as appropriate to the application) fertilized with separated and unseparated spermatozoa are similar, and events during the first cell cycle are timed similarly for separated and unseparated spermatozoa. However, with conventional procedures, blastocyst production with separated spermatozoa can be 70%-90% of controls with spermatozoa that have not been separated. For example, development to blastocysts has been shown to be 17% with bovine oocytes inseminated with separated spermatozoa, compared with >25% which might be expected with IVF using unseparated spermatozoa.

Several factors may contribute to these results. During preparation for separation, spermatozoa can be subjected to conditions that may affect IVF procedures differently from in-vivo conditions. One factor can be the staining of frozen sperm with Hoechst 33342 at 35° C which appears to cause a decline in motility of spermatozoa derived from previously frozen sperm. Another factor, can be the physical forces the spermatozoa are subject to during the separation process. As but one example, in flow cytometric separation of spermatozoa,

spermatozoa exit the flow cytometer at nearly 100 km/h before impacting on the surface of the collection medium. During transit through the flow cytometer spermatozoa are also subjected to laser light at an intensity of over 100mW. While the transit time may only be 1-2 $\mu$ sec, this may affect the spermatozoa's DNA, and thus, also effect subsequent embryonic development.

5 The process of separating sperm with flow cytometrically also results in a highly diluted sample, 600,000 spermatozoa/mL or less, and subsequent centrifugation steps are necessary to provide concentrated spermatozoa suitable for insemination.

As to the problems with conventional techniques of IVF using separated spermatozoa, and specifically separated spermatozoa, stained spermatozoa, or spermatozoa that are from

10 previously frozen sperm, the instant invention addresses each of the above-mentioned concerns.

#### SUMMARY OF THE INVENTION

Accordingly, one of the broad objects of particular embodiments of the invention can be to provide devices, compositions and methods to that promote cleavage and improve the quality of embryos generated using IVF with spermatozoa separated into X-chromosome bearing and

15 Y-chromosome bearing populations.

Another broad object of particular embodiments of the invention can be to provide to provide devices, compositions, and methods that promote cleavage and improve quality of embryos generated using IVF with spermatozoa that are derived from previously frozen sperm.

Another broad object of particular embodiments of the invention can be to provide to

20 provide devices, compositions, and methods that promote cleavage and improve quality of embryos generated using IVF with spermatozoa that have previously been stained with a DNA binding fluorochrome.

Another broad object of particular embodiments of the invention can be to provide to provide media for embryonic culturing that can contain non-essential amino acids.

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

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an embodiment of the invention in which spermatozoa from a fresh or  
5 a previously frozen-thawed sperm sample are stained.

Figure 2 shows an embodiment of the invention for separating stained spermatozoa in to X-chromosome bearing and Y-chromosome bearing populations.

Figure 3 shows another view of an embodiment of the invention for separating stained spermatozoa in to X-chromosome bearing and Y-chromosome bearing populations.

10 Figure 4 presents a bar graph showing percentages of oocytes developing to the two pronuclei stage at 20 hours after insemination with either separated or unseparated spermatozoa.

Figure 5 presents a bar graph showing percentages of oocytes at various stages at 25 hours after insemination.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

15 As mentioned earlier, the present invention includes a variety of aspects which may be combined in different ways. As can be easily understood, the basic concepts of the present invention may be embodied in a variety of ways. It involves devices, compositions, and methods to accomplish IVF with spermatozoa derived from previously frozen sperm, with spermatozoa that have been previously stained, or with separated spermatozoa which can result in higher  
20 quality embryos. In this patent application, the IVF apparatus, compositions, and methods are disclosed as part of the results shown to be achieved by the various devices described and as steps which are inherent to utilization. They are simply the natural result of utilizing the devices

as intended and described. In addition, while some devices are disclosed, it should be understood that they 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.

5        Embodiments of the invention can include fresh spermatozoa, or spermatozoa from frozen-thawed sperm of numerous species of mammals. The invention should be understood not to be limited to the species of mammals cited by the specific examples within this patent application. Embodiments of the invention, for example, may include fresh spermatozoa or spermatozoa from frozen-thawed sperm of animals having commercial value for meat  
10 production such as cattle, pigs, sheep, or the like (naturally the mammals used for meat production may vary from culture to culture). It may also include fresh spermatozoa or spermatozoa from frozen-thawed sperm from individuals having uncommon attribute(s), such as morphological characteristics including weight, size, or conformation, or other desired characteristics such as speed, agility, intellect, or the like. It may include fresh or frozen-thawed  
15 sperm from deceased donors, or from rare or exotic mammals, such as zoological specimens or endangered species. The invention may even be used in the context of fresh spermatozoa or spermatozoa from frozen-thawed human sperm.

Now referring to Figure 1, in some embodiments of the invention, Hoechst 33342 stain  
(2) can be added to bovine spermatozoa contained in frozen-thawed sperm (1) to establish a  
20 concentration of 224 $\mu$ M. The incubation time of the spermatozoa contained in the frozen-thawed sperm (1) with the stain (2) can be about 190 minutes. In another embodiment of the invention, the stain (2) can be added to the bovine sperm (1) to establish a concentration of 2240 $\mu$ M and then incubated for about 60 minutes. Frozen-thawed sperm treated in either manner can improve the resolution of X-chromosome bearing from Y-chromosome bearing  
25 spermatozoa. Understandably, from application to application (such as frozen-thawed sperm from different species) the amount of incubation time and the specific concentration of stain can be adjusted to optimize the resolution of the X-chromosome bearing from Y-chromosome bearing

spermatozoa.

With respect to the cleavage rates of inseminated oocyte(s), the increase in stain concentration up to at least 10X does not appear to have a depressive effect on either cleavage or embryonic development. Higher stain concentrations may actually be beneficial with respect to certain applications because the length of incubation time may be decrease improving percent cleavage. From application to application length of incubation time can be adjusted to optimize cleavage results or embryonic development, as desired.

Now referring to Figures 2 and 3, a flow cytometer embodiment of the invention is shown which includes a cell source (3) which acts to establish or supply stained spermatozoa or other type of stained cells to be analyzed by the flow cytometer. The cells are deposited within a nozzle (4) in a manner such that the cells are surrounded by a sheath fluid (5). The sheath fluid (5) is usually supplied by some sheath fluid source (6) so that as the cell source (3) supplies its cells, the sheath fluid (5) is concurrently fed through the nozzle (4). In this manner it can be easily understood how the sheath fluid (5) 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 (4) and exit at the nozzle orifice (7). By providing some type of oscillator (8) which may be very precisely controlled through an oscillator control (9), pressure waves may be established within the nozzle (4) and transmitted to the fluids exiting the nozzle (4) at nozzle orifice (7). Since the oscillator (9) thus acts upon the sheath fluid (5), the stream (10) exiting the nozzle orifice (7) eventually and regularly forms drops (11). Because the cells are surrounded by a sheath fluid environment, the drops (11) may contain within them individually isolated (generally) cells or other items.

Since the drops (11) generally contain isolated cells, the flow cytometer can distinguish and separate droplets based upon whether or not the appropriate cell or cells is/are contained within the drop. This is accomplished through a cell sensing system (12). The cell sensing system involves at least some type of sensor (13) which responds to the cells contained within

each drop (11) as discussed at length in the seminal work (no pun intended) by Larry Johnson, namely, U.S. Patent No. 5135759, hereby incorporated by reference. As the Johnson patent explains for spermatozoa or sperm cells, although the staining and separation inventions can be understood to be used with a variety of frozen-thawed cells, the cell sensing system (13) may  
 5 cause an action depending upon the relative presence or relative absence of the bound fluorochrome which may be excited by some stimulant such as the laser exciter (14). While each type of sperm cell can be stained by the stain or fluorochrome, as described above, the differing length of the X-chromosome and the Y-chromosome causes different amounts of stain to be bound. Thus, by sensing the degree of fluorescence emitted by the fluorochrome upon excitation  
 10 it is possible to discriminate between X-bearing spermatozoa and Y-bearing spermatozoa by their differing emission levels.

In order to achieve the ultimate separation and isolation of the appropriate cells, the signals received by sensor (14) are fed to some type of sorter discrimination system (15) which very rapidly makes the decision and can differentially charge each drop (11) based upon whether  
 15 it has decided that the desired cell does or does not exist within that drop (11). In this manner the separation or discrimination system (15) acts to permit the electrostatic deflection plates (16) to deflect drops (11) based on whether or not they contain the appropriate cell or other item. As a result, the flow cytometer acts to sort the cells by causing them to land in one or more collectors (17). Thus by sensing some property of the cells or other items the flow cytometer can  
 20 discriminate between cells based on a particular characteristic and place them in the appropriate collector (17). In the system presently used to sort spermatozoa, the X-bearing spermatozoa droplets are charged positively and thus deflect in one direction, the Y-bearing spermatozoa 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  
 25 or the like.

Now referring to Figure 3, the process can be even further understood. As shown in that figure, the nozzle (4) emits a stream (10) which because of the oscillator (8) (not shown in

Figure 3) forms drops (11). Since the cell source (3) (not shown in Figure 3) may supply sperm cells (1) which have been stained according the invention, the light stimulation by laser exciter (13) is differentially determined by sensor (14) so that the existence or nonexistence of a charge on each drop (11) as it separates from stream (10) can be controlled by the flow cytometer. This control results in positively charged, negatively charged, and uncharged drops (8) based upon their content. As shown in Figure 3, certain drops are shown as deflected drops (18). These deflected drops (18) are those containing sperm cells (1) differentiated by bearing an X-chromosome or a Y-chromosome. Separated sperm are then deposited in the appropriate collector (17) for later use.

10 While the above description focuses on the separation of spermatozoa with flow cytometer, separation of X-chromosome bearing spermatozoa and Y-chromosome bearing spermatozoa based upon the difference in measurable fluorescent emission may also include numerous other technologies such as liquid chromatography, gel electrophoresis, and other technologies that similarly excite the amount of bound fluorochrome to differentiate between X  
15 chromosome bearing spermatozoa and the Y chromosome bearing spermatozoa.

Embodiments of the invention can also include IVF insemination samples for utilization in IVF insemination protocols or kits including compositions of culture media similar to those described.

#### EXAMPLE 1.

20 This study sought to determine differences among bulls in fertilization and embryonic development rates of oocytes fertilized by flow-cytometrically sorted as well as unsorted spermatozoa. A total of 1949 bovine oocytes and sperm from 5 bulls (2-3 replicates each bull) were examined. Oocytes, aspirated from slaughterhouse ovaries, were matured in TCM199 supplemented with 10% fetal calf serum plus hormones (15 ng FSH, 1  $\mu$ g LH, 1  $\mu$ g  $E_2$ /ml) for  
25 22-24 h at 39°C, in 5% CO<sub>2</sub> in air. Flow cytometrically-sorted X- or Y-chromosome-bearing spermatozoa as well as unsorted control spermatozoa from the same bull were cryopreserved for

later use with IVF. The procedures for IVF and culture have been previously described in Theriogenology, 52, 1393-1405 (1999), hereby incorporated by reference herein. Presumptive zygotes were removed from culture and placed in chemically-defined medium (CDM-1) as discussed in Journal Animal Science, 78, 152-157 (2000), hereby incorporated by reference  
 5 herein, 6-7 hours after insemination and cultured for 65-66 hours. Embryos which had cleaved were further cultured 96 hours in CDM-2 containing 0.12 IU insulin/mL. Blastocysts were morphologically graded according to the size of inner cell mass and stained with Giemsa to determine cell numbers on D7 after insemination. Data were analyzed by Chi Square.

There was a reduction ( $P<0.01$ ) in cleavage rate between sorted and unsorted  
 10 spermatozoa from bulls, H001 and H003: 46 vs 76 and 35 vs. 67%. No difference was found in cleavage rates between sorted and unsorted spermatozoa from the other 3 bulls where cleavage rates ranged from 66 to 78% for sorted sperm and 69 to 88% for unsorted sperm. Sperm from bull H001 resulted in very poor embryo production per oocyte: 11% for sorted and 16% for unsorted spermatozoa. Blastocyst production from bull H003 was very low (6%,  $P<0.01$ ) with  
 15 sorted spermatozoa while production with unsorted spermatozoa was normal (26%). Blastocyst development was similar with sorted and unsorted spermatozoa from the other bulls ranging from 20 to 26% for sorted and from 24 to 37% for unsorted spermatozoa. Proportions of grade 1 blastocysts obtained from sorted and unsorted sperm were similar in 4 of 5 bulls and higher for sorted sperm for bull H001 ( $P<0.01$ ). Mean cell numbers in expanding blastocysts were  
 20 similar among bulls and sperm treatments, ranging from 126 to 167 for sorted sperm and from 120 to 164 for unsorted spermatozoa.

#### EXAMPLE 2.

Sperm from four bulls were used (five replicates per bull) to compare fertilization and  
 25 embryonic development of oocytes inseminated with separated spermatozoa (Table 1). Both CDM and SOF systems were used to culture embryos, half in each.

Table 1.

|   | Sperm Type      | No. oocytes | No. cleaved (%)       | No. blastocysts (%)   |
|---|-----------------|-------------|-----------------------|-----------------------|
|   | Sorted-Fresh    | 792         | 523 (66) <sup>a</sup> | 128 (16) <sup>a</sup> |
|   | Unsorted-Fresh  | 721         | 544 (76) <sup>b</sup> | 172 (24) <sup>b</sup> |
|   | Sorted-Frozen   | 554         | 394 (71)              | 100 (18) <sup>a</sup> |
| 5 | Unsorted-Frozen | 566         | 423 (75)              | 140 (25) <sup>b</sup> |

<sup>a,b</sup> Percentages within columns with different superscripts differ ( $P>.01$ ),  $\chi^2$

While separating the spermatozoa appears to have had a small effect on rates of cleavage, the percentage of oocytes developing to blastocysts was only about 70% of the rate with non-separated spermatozoa. Nearly identical results occurred with separated fresh spermatozoa compared to separated then frozen spermatozoa.

It further appears that there is a one-half to one-day delay of embryonic development when oocytes are inseminated with separated spermatozoa and co-cultured with granulosa cell monolayers compared to insemination with unseparated spermatozoa (Tables 2 and 3).

Table 2.

| 15 | Sperm Type | No. oocytes | % cleaved | % blastocysts/oocyte | % blastocysts/oocyte |    |    |
|----|------------|-------------|-----------|----------------------|----------------------|----|----|
|    |            |             |           |                      | D7                   | D8 | D9 |
|    | Sorted     | 396         | 78        | 17                   | 18                   | 51 | 31 |
|    | Unsorted   | 376         | 86        | 35                   | 44                   | 41 | 15 |

Table 3.

| 20 | Sperm Type | No. oocytes | % cleaved | % blastocysts/oocyte | % becoming blastocytes |    |    |     |
|----|------------|-------------|-----------|----------------------|------------------------|----|----|-----|
|    |            |             |           |                      | D7                     | D8 | D9 | D10 |
|    | X - sperm  | 157         | 78        | 26                   | 29                     | 49 | 15 | 7   |
|    | Y - sperm  | 162         | 74        | 28                   | 30                     | 54 | 13 | 2   |

Fewer blastocysts were produced per oocyte with separated spermatozoa, and only about 18% of embryos that eventually developed to blastocysts did so on day seven (7) compared with 44% with unseparated spermatozoa. The majority of blastocysts (82%) developed on days eight (8) and nine (9) with separated spermatozoa (Table 2).

5 When X-chromosome bearing spermatozoa and Y-chromosome bearing spermatozoa rather than bulk separated live sperm were used, the majority of embryos developed to blastocysts on day eight (8) (Table 3). The speed of development of embryos from Y-chromosome bearing spermatozoa compared to development of embryos from X-chromosome bearing spermatozoa was not detected.

10 Embryonic development of ova fertilized with separated spermatozoa and unseparated spermatozoa was then compared. A total of 1143 oocytes in five replicates were fertilised with separated or unseparated spermatozoa in either conventional TALP medium or CDM-1 medium. Bulk separated fresh sperm from two bulls were used. Ten to 15 oocytes per replicate from each of four treatment groups were fixed every 5 hours up to 25 hours following insemination and  
15 stained with acetoorcein for examination of sperm penetration and pronuclear formation.

The percentages of oocytes developing to the two pronuclei stage at 20 hours after insemination either with separated or unseparated spermatozoa in either CDM or Fert-TALP medium are present in Figure 4. There was no significant difference ( $P>.05$ ) in the rate of pronuclei formation between oocytes inseminated either with separated (56%) and unseparated  
20 (68%) spermatozoa, or between Fert-TALP (63%) and CDM medium (62%). Data for 5, 10, 15 hour post-insemination are not present as no difference was observed between any of the treatments at these times.

There was also no significant difference between separated and non-separated spermatozoa in percentages of oocytes reaching various stages at 25 hours after insemination  
25 as shown by Figure 5. Thus, a fertilization or cell cycle delay with separated spermatozoa was

not found up to 25 hours following insemination. At 25 hours development was more advanced with both separated and non-separated when fertilization occurred in CDM medium than in Fert-TALP medium (38% and 30% versus 0% and 0% 2-cell embryos;  $P < 0.05$ ).

A further experiment was designed to study various IVF culture treatments with 5 separated sperm that might increase speed of embryonic development. The 4 x 2 factorial experimental design included four fertilization media: 1) Fert-TALP, 2) Fert-TALP-NEAA (non-essential amino acids from Eagle's Medium, 3) CDM-1, and 4) SOF and two culture media: 1) CDM, and 2) SOF. Separated spermatozoa from frozen sperm from a single bull were used. A total of five replicates were carried out, with the exception that only three replicates were 10 conducted to examine cleavage at 24 hours and at 72 hours (Table 4). Cleavage and embryonic development were evaluated at 24, 72, 168, and 192 hours after insemination. Quality of embryos was recorded. Results are presented in Tables 4-6.

Table 4.

|    | Media            | No. oocytes | % cleavage | % 2-cell at 24 h | %8-cell at 72 h |
|----|------------------|-------------|------------|------------------|-----------------|
| 15 | Fert-TALP        | 168         | 76         | 6 <sup>a</sup>   | 66              |
|    | Fert-TALP + neaa | 176         | 71         | 26 <sup>b</sup>  | 67              |
|    | CDM              | 167         | 89         | 75 <sup>c</sup>  | 70              |
|    | SOF              | 145         | 86         | 49 <sup>d</sup>  | 69              |

<sup>a,b,c,d</sup> Means with different superscripts differ ( $P < .05, \chi^2$ ).

20 Table 5.

| Media         | No. oocytes | % blastocysts/oocyte |    | % Grade 1 blastocysts/ total blastocysts |
|---------------|-------------|----------------------|----|------------------------------------------|
|               |             | Total                | D7 |                                          |
| Fert-TALP     | 326         | 20                   | 17 | 52 <sup>a,c</sup>                        |
| Fert-TALP -aa | 221         | 20                   | 17 | 68 <sup>b</sup>                          |
| CDM           | 332         | 22                   | 18 | 61 <sup>b,c</sup>                        |
| 5 SOF         | 321         | 21                   | 17 | 64 <sup>b,c</sup>                        |

<sup>a,b,c</sup> Percentages without common superscript differ (P<.05).

Table 6.

| Media  | No. oocytes | % cleavage | % blastocysts/oocyte |    | % Grade 1 blastocysts/ total blastocysts |
|--------|-------------|------------|----------------------|----|------------------------------------------|
|        |             |            | Total                | D7 |                                          |
| CDM    | 596         | 78         | 21                   | 19 | 66                                       |
| 10 SOF | 604         | 72         | 21                   | 16 | 56                                       |

No significant differences.

Fewer oocytes (P<0.05) fertilized in Fert-TALP developed to the 2-cell stage by 24 hours than in any other media. Notably, the vast majority of oocytes (75%) fertilized in CDM medium cleaved to 2-cell stage by this time (Table 4). By 72 hours post-insemination, there was no  
15 difference between any of the media, possibly due to the long 8-cell stage cell cycle.

There was no difference between any of the media on rate of development to blastocysts (Table 5). However, there was a significant difference in quality of embryos between Fert-TALP and Fert-TALP +aa.

Progression of early bovine embryonic development using separated sperm are similar  
20 to studies with in-vivo or in-vitro cleavage of oocytes fertilized with unseparated spermatozoa. In the cow the first in-vivo cleavage occurs at 24-28 hours following ovulation, and the first in-

vitro cleavage takes place at 24-48 hours after insemination.

Earlier cleavage occurred with oocytes fertilized in CDM, SOF, and Fert-TALP + aa medium than in conventional Fert-TALP medium. This can be because CDM, SOF, and Fert-TALP +aa media all contain non-essential amino acids, which may play a role in how quickly  
5 spermatozoa penetrate oocytes, of in the length of the first cell cycle.

As can be easily understood from the foregoing, the basic concepts of the present invention may be embodied in a variety of ways. It involves both IVF techniques as well as devices to accomplish IVF utilizing spermatozoa separated into X-chromosome bearing and Y-chromosome bearing populations. In this application, the IVF techniques are disclosed as part  
10 of the results shown to be achieved by the various devices described and as steps which are inherent to utilization. They are simply the natural result of utilizing the devices as intended and described. In addition, while some devices are disclosed, it should be understood that these not only accomplish certain methods but also can be varied in a number of ways. Importantly, as to all of the foregoing, all of these facets should be understood to be encompassed by this  
15 disclosure.

The discussion included in this provisional 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  
20 actually be representative of a broader function or of a great variety of alternative or equivalent elements. Again, these are implicitly included in this disclosure. Where the invention is described in 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  
25 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 and may be relied upon when drafting the claims for the full patent application. It should be understood that such language changes and broad claiming will be accomplished when the applicant later (filed by the required deadline) seeks a patent filing based on this provisional filing. The subsequently filed, non-provisional patent application will seek examination of as broad a base of claims as deemed within the applicant's right and will be designed to yield a patent covering numerous aspects of the invention both independently and as an overall system.

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 "separated spermatozoa" should be understood to encompass disclosure of the act of "separating spermatozoa" -- whether explicitly discussed or not -- and, conversely, were there effectively disclosure of the act of "separating spermatozoa", such a disclosure should be understood to encompass disclosure of a "separated spermatozoa" and even a "means for separating

spermatozoa” Such changes and alternative terms are to be understood to be explicitly included in the description.

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  
 5 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  
 10 References To Be Incorporated By Reference In Accordance With The Provisional Patent 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 the  
 15 applicant(s).

Thus, the applicant(s) should be understood to claim at least: i) each of the 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 and methods, iv) those alternative designs which accomplish each of the functions shown as are disclosed and described,  
 20 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, viii) the resulting products produced by such systems or components, and ix) methods and apparatuses substantially as described hereinbefore and with  
 25 reference to any of the accompanying examples, and x) the various combinations and permutations of each of the elements disclosed.

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  
5 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.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO  
DIRECCIÓN DE NUEVAS CREACIONES

**Radicación No.** 03-54828  
**Clasificación Internacional (IPC):** A61B, A01K 67/00, 67/033, C12N 5/00, 5/02, 15/00.  
**Concepto Técnico No.** 3424

|                                 |                                     |                               |                                     |
|---------------------------------|-------------------------------------|-------------------------------|-------------------------------------|
| Patente de Invención            | <input checked="" type="checkbox"/> | Patente de Modelo de Utilidad | <input type="checkbox"/>            |
| Vía Nacional                    | <input type="checkbox"/>            |                               |                                     |
| Vía PCT (fase nacional)         | <input checked="" type="checkbox"/> | Cap. I                        | <input type="checkbox"/>            |
|                                 |                                     | Cap. II                       | <input checked="" type="checkbox"/> |
| No. Sol. Internacional          | PCT/US01/45023                      |                               |                                     |
| Fecha de Presentación Internal. | 29/11/2001                          |                               |                                     |

Como resultado del examen definitivo, realizado con fundamento en el artículo 48 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

1. Documentos estudiados

|                              |                                       |                                                                     |
|------------------------------|---------------------------------------|---------------------------------------------------------------------|
| <u>Descripción:</u>          | Folios: 58 a 96                       | tal como se radicó inicialmente                                     |
| <u>Reivindicaciones:</u>     | Folios: 97 a 117<br>Folios: 222 a 228 | tal como se radicó inicialmente<br>tal como se modificó el 23/02/10 |
| <u>Prioridad:</u>            | Fecha<br>País<br>Número               | 29/11/2000<br>US<br>60/253,785                                      |
| <u>Rta. del solicitante:</u> | Folios: 239 a 245                     | como se radicó el 21/09/2010                                        |
| <u>Anexos:</u>               | Folios: 246 a 261                     | como se radicó el 21/09/2010                                        |

2. Objeto de la invención

- Título de la solicitud (folio 1): "Disposición para separar espermatozoides congelados en poblaciones que contienen cromosomas X y cromosomas Y".
- La presente solicitud se refiere a un método para diferenciar espermatozoides y permitir su separación en poblaciones que contienen cromosomas X y cromosomas Y.
- El problema planteado en esta solicitud es la necesidad de separar espermatozoides que contienen cromosomas X de espermatozoides que contienen cromosomas Y que cada población resultante contenga un

número significativo de espermatozoides separados de forma correcta e uniforme.

- Para solucionar este problema técnico, la solicitud en estudio proporciona un método para la tinción de células de esperma recolectada de mamíferos.
- La Clasificación Internacional de Patentes (IPC) es A61B, A01K 67/00, 67/033, C12N 5/00, 5/02, 15/00.

3. Determinación del Estado de la Técnica

La fecha que se ha tenido en cuenta para determinar el Estado de la Técnica es:

Fecha de presentación de la solicitud prioritaria: 29/11/2000.

Teniendo en cuenta la fecha indicada se realizó la búsqueda en el estado de la técnica de documentos relacionados con la solicitud encontrándose los siguientes documentos más relevantes:

| Nº | Documento    | Título                                                                           | Fecha de publicación* |
|----|--------------|----------------------------------------------------------------------------------|-----------------------|
| D1 | US 6.149.867 | Sheath fluids and collection systems for sex-specific cytometer sorting of sperm | 21/11/2000            |

El Documento del estado de la técnica D1 con número de publicación US 6.149.867 por Seidel *et al.*, se refiere a 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.

4. Análisis de patentabilidad (artículo 14 D486)

Nivel inventivo (artículo 18 D486)

La solicitud en estudio reclama (reivindicación 1 y dependientes) un método para la tinción de células de esperma recolectada de mamíferos que comprende las etapas de:

- a) Recolectar semen de un mamífero macho;
- b) Congelar dicho semen que contiene dichas células de esperma;
- c) Descongelar dicho semen que contiene dichas células de esperma;
- d) Incubar células de esperma contenidas dentro de dicho semen en una concentración micro-molar de colorante Hoechst 33342 entre 40 y 2500;
- e) Establecer la temperatura a la cual dichas células de esperma en dicha concentración de colorante Hoechst 3342 son incubadas entre 30°C y 40°C y;
- f) Ajustar un tiempo de duración en que dichas células de esperma son incubadas con dicha concentración de colorante Hoechst 3342 entre 50 minutos y 200 minutos.

El documento del Estado de la Técnica más próximo es D1 (ver folios: 191 – 198) que enseña una forma de separar cromosomas X de cromosomas Y mediante un citómetro de flujo. En el ejemplo 1 señala la forma de tinción de células de esperma mediante su incubación a  $38\ \mu\text{M}$  de colorante Hoechst 3342 por una hora a  $34^\circ\text{C}$  (columna 15 líneas 2- 8). De esta forma, se alcanzó una pureza de 90% apx. (línea 9). Los experimentos llevados a cabo en D1 (ejemplos 1, 2 y 3) involucran el congelamiento y descongelamiento del semen con fines de su transporte, dicho mecanismo por lo tanto es normal en el estado de la técnica antes o después de la tinción.

La única diferencia evidente es que la concentración empleada en D1 obedece a una concentración menor a los valores empleados por el solicitante, aunque muy cercana al límite inferior del intervalo reclamado ( $40 - 2500\ \mu\text{M}$ ). Sin embargo, dicha diferencia no evidencia un efecto sorprendente en el estado de la técnica ya que la eficiencia del proceso en términos de pureza de esperma con cromosomas X era la misma (90%) como se observó anteriormente.

Puesto que D1 divulgaba previamente la necesidad de separar espermatozoides que contienen cromosomas X de espermatozoides que contienen cromosomas Y, y motivaban que cada población resultante tuviera un número significativo de espermatozoides separados de forma correcta e uniforme, una persona conocedora de la materia motivada por tal sugerencia lograría métodos presentados en la solicitud basándose en las enseñanzas de D1.

De esta forma se establece que las reivindicaciones 1 a 20 NO cumplen con el requisito de Nivel Inventivo.

## 5. Respuesta a los argumentos del solicitante:

- El solicitante argumenta sobre el nivel inventivo de la presente solicitud que la anterioridad D1 enseña una forma de separar cromosomas X de cromosomas Y, pero en ningún momento hace referencia a la separación de semen previamente congelado alegando en su favor que en los ejemplos 1 a 4 no se encontraba la información necesaria para que una persona versada en la materia se viera motivada derivar de manera obvia la presente solicitud. En síntesis, no hay ningún indicio o sugerencia en D1 para separar esperma descongelado que había sido previamente congelado.

Como se ha determinado en el análisis de patentabilidad, las diferencias de la solicitud y la anterioridad radican en la separación de esperma habiendo sido previamente congelado y descongelado, o de semen fresco, por lo tanto, el problema que el solicitante intenta plantear es si la metodología podía ser aplicable con éxito a semen de una procedencia con condiciones diferentes.

Ante este particular, debido a que no se evidencia un efecto técnico sorprendente, ni se encuentra un sustento que impidiera separar mediante una metodología similar como la ya observada esperma de variada procedencia, la solicitud continúa careciendo de altura inventiva. 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 que en 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.

Por tal razón no es posible aceptar los argumentos del solicitante.

- El solicitante menciona que el método reivindicado proporciona una solución, la cual es muestra del nivel inventivo involucrado y que permite la separación de esperma congelado descongelado, lo que no solo no había sido logrado anteriormente, sino que la persona versada en la materia consideraba que no era posible lograrlo.

Dicho argumento prueba efectivamente que es posible separar semen previamente congelado y descongelado, pero el procedimiento para realizarlo es el mismo que el presentado por el documento del estado de la técnica señalado por este Despacho. Según este argumento, dicha característica resultaría ser un objetivo 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 para el producto final. Por esta razón, no es posible aceptar dicho argumento.

- El solicitante menciona que con el fin de lograr la suficiente resolución en cada una de las fluorescencias de las células de esperma, cada célula en la muestra debe estar uniformemente teñida.

Ante este argumento, se hace énfasis en lo mencionado en el análisis de patentabilidad por cuanto, los valores empleados de tinción de concentración de colorante, tiempo, y temperatura son muy similares, siendo de hecho, menores en la anterioridad y obteniéndose una buena calidad (90%) en la viabilidad. Por tanto, el problema que se plantea en este argumento ya estaba resuelto en D1 como se observó en el análisis de patentabilidad.

- El solicitante argumenta que las células de esperma son delicadas y que elevadas concentraciones de colorante y elevados tiempos de tinción tienen un impacto negativo en su viabilidad. Argumenta además que la tabla 1 presenta una relación de los tiempos de tinción y las concentraciones de tintura para alcanzar alta pureza en la separación de células de esperma mientras se mantiene su viabilidad.

Aunque no es fácil la interpretación de los datos de la tabla 1 por cuanto las unidades de concentración no se encuentran debidamente anotadas, se entiende que los datos observados en micro molar son mayores que los reportados por la anterioridad D1, lo cual si bien puede no ser comparable, tampoco brinda un efecto sorprendente sobre la viabilidad de las células de esperma, por cuanto implicaría que la solicitud presenta ese mismo impacto negativo que argumenta sobre las células de esperma. Igualmente los tiempos requeridos de tinción son mayores o iguales a una hora, por lo que no se considera una ventaja sobre la anterioridad.

- Por las razones anteriores, este Despacho considera que las objeciones realizadas en el Concepto Técnico 1395 no fueron subsanadas, y los argumentos del solicitante no son aceptados.

6. Conclusión:

En consecuencia y por todo lo expuesto anteriormente, se recomienda **Negar** las reivindicaciones: 1 a 20 (folios: 222 - 228)

Bogotá, D.C.,

|                                     |                                                |
|-------------------------------------|------------------------------------------------|
| <b>CONCEPTO TECNICO</b><br>No. 3424 | <b>EXAMINADOR TECNICO</b><br>Código No. 202089 |
|-------------------------------------|------------------------------------------------|

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