

## DESCRIPTION

### Title of the Invention

Method of Detecting Nucleic Acid Cleavage Contained in Single  
5 Cell

### Cross Reference to Related Application

[0001] The present application claims the benefit of priority of Japanese  
Patent Application No. 2012-166668, filed on July 27, 2012, the entire  
10 contents of which are incorporated herein by reference.

### Technical Field

[0002] The present invention relates to a method of detecting nucleic  
acid cleavage in a single cell, a method of manufacturing a cell that is free  
15 from nucleic acid cleavage and a kit for executing either or both of these  
methods.

### Background Art

[0003] DNA damages extend over a wide range including double strand  
20 breaks, cleavages only on one side, oxidative damages and base  
modifications by chemical substances, of which double strand breaks are  
endogenously most difficult to repair and entail the highest cell lethality  
because a single strand break gives rise to a serious problem if an  
important gene is damaged by the break.

**[0004]** Body cells possess multiple DNA repair mechanism. However, sperm loses the DNA repair capacity in and after the anaphase of the second meiotic division period. If an egg fertilizes with a sperm that having unrepaired or imperfectly repaired DNA, it leads to pregnancy losses such as chromosomal abnormality and an increase in the miscarriage rate. Therefore, the critical threshold to DNA double strand breaks is very low and it is desired to establish a method that can detect DNA cleavages in the initial stages from the view of sperm quality management in the field of assisted reproduction technology (ART).

**[0005]** Known techniques of detecting DNA cleavages include sperm chromatin dispersion tests, the TUNEL method and the COMET method.

**[0006]** With a sperm chromatin dispersion test, the sperm embedded in agarose is subjected to cytolysis, or cell dissolution, and subsequently the DNA fragments that have diffused around are observed (see Non-Patent Document 1 listed below, which document is cited herein for the purpose of supporting the disclosure of the present invention). The TUNE method is a microscopic observation of DNA fragments that are labeled with a fluorescent dye (see Non-Patent Documents 2 and 3 listed below, which documents are cited herein for the purpose of supporting the disclosure of the present invention).

**[0007]** The COMET method is currently the most popular method of detecting DNA cleavage in a single cell, to date, a large number of reports using this method have been published (see Non-Patent Documents 4 through 6 listed below, which documents are cited herein for the purpose of supporting the disclosure of the present invention). With the COMET method, the sperm embedded in agarose is subjected to cytolysis and, thereafter, the DNA fragments are isolated by electrophoresis. It is said that the COMET method allows not only DNA double-strand breaks but also single-strand breaks by an alkali treatment.

## Prior Art Documents

### Non-Patent Documents

[0008]

#### 5 Non-Patent Document 1:

Fernandez JL, Muriel L. RiveroMT, Goyanes V. VazquezR, et al. (2003). The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. JAndrol 24: 59-66.

#### Non-Patent Document 2:

10 Dominguez-Fandos D, Camejo MI, Bal lesca JL, Ol iva R (2007) Human sperm DNA fragmentation: correlation of TUNEL results as assessed by flow cytometry and optical microscopy. Cytometry Part A 71A: 1011-1018.

#### Non-Patent Document 3:

15 Sharma RK, Sabanegh E, Mahfouz R, Gupta S, Thiagarajan A, et al. (2010) TUNEL as a rest for sperm DNA damage in the evaluation of male infertility. Urology 76: 1380-1386.

#### Non-Patent Document 4:

20 Hughes CM, McKelvey-Martin VJ, Lewis SE (1999) Human sperm DNA integrity assessed by the Comet and ELISAssays. Mutagenesis 194: 71-75.

#### Non-Patent Document 5:

25 Morris ID, Ilott S, Dixon L, Brison DR (2002) The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. Hum Reprod 17: 990-998.

Non-Patent Document 6: Simon L, Lutton D, McMunus J, Lewis SE (2011) Sperm DNA damage measured by the alkaline Comet Assay as an

independent predictor of male infertility and in vitro fertilization success.  
Fertil Steril 95: 652-657.

## **Disclosure of the Invention**

### **5 Problems to Be Solved by the Invention**

[0009] However, the sperm chromatin dispersion test, the TUNEL method and the COMET method are all insufficient and unsatisfactory as methods of detecting DNA cleavage in the initial stages thereof.

10 [0010] More specifically, the sperm chromatin dispersion test is capable of detecting a situation where DNA fragmentation has progressed to a certain extent and hence cannot detect the initial stages of DNA cleavage. With the TUNEL method, a certain amount of DNA fragments needs to be labeled with a chromophore for the purpose of fluorescent sensing under microscope. Thus, the TUNEL method is also unsuited for detecting DNA  
15 cleavage in the initial stages thereof.

[0011] On the other hand, the COMET method is a capable of detecting particular DNA fragments produced as a result of advanced stage of fragmentation and hence it cannot detect initial stage with several sites of DNA cleavage in a single cell. In other words, the COMET method does  
20 not provide a satisfactory level of sensitivity for detecting DNA cleavage in the initial stages thereof.

[0012] In view of the above-identified problems of the prior art methods, it is therefore the object of the present invention to provide a novel method of detecting DNA cleavage in the initial stages thereof.

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### **Means for Solving the Problems**

[0013] In eukaryotic cells, the DNA in the nucleus is bound to the nucleoproteins and anchored to the nuclear membrane by means of structural proteins that is referred to nuclear matrix. Beside, in prokaryotes, DNA is bound to the nucleoid protein.

5 [0014] As a result of intensive research efforts, the inventor of the present invention found (a) that the DNA in the cell nucleus can be separated from the nucleoprotein and the nuclear matrix by embedding the cell in a molten gel, to which proteolytic enzyme has been added in an inactive state, solidifying the gel, subsequently activating the proteolytic  
10 enzyme in the gel and digesting the cell and (b) that a long chain DNA fragment that is found in the initial stages of DNA cleavage in the nucleus contained in a single cell can be detected by causing electrophoresis to take place on the separated DNA in the nucleus by means of pulsed field gel electrophoresis to achieve the above object of the invention.

15 [0015] Particularly, the inventor of the present invention looked into detecting various long chain DNA fragments with a higher degree of sensitivity. Firstly, the inventor of the present invention paid attention to that the lengths of DNA fragments that are obtained by means of one-dimensional pulsed field gel electrophoresis are shorter than the  
20 length of the DNA in the cell nucleus and presumed that there may be long chain DNA fragments that cannot be observed by means of one-dimensional pulsed field gel electrophoresis. Thus, the inventor of the present invention tried to realize two-dimensional pulsed field gel electrophoresis instead of one-dimensional pulsed field gel electrophoresis.  
25 However, there were no noticeable significant changes in the results of observation by electrophoresis when the experiment technique of one-dimensional pulsed field electrophoresis was simply applied to two-dimensional pulsed field gel electrophoresis.

[0016] Thus, the inventor of the present invention intensively looked in

the conditions of electrophoresis including the agarose concentration, the composition of the cell lysate, the angle between the two pairs of electrodes to be used for electrophoresis, the electrophoresis voltage, the timing of electrode switching, the composition of the solution for electrophoresis and  
5 the angle of rotation of the glass slide that is smeared with cells. As a result, the inventor of the present invention succeeded in detecting long chain DNA fragments by means of two-dimensional pulsed field gel electrophoresis that could never be detected by means of one-dimensional pulsed field gel electrophoresis. This is a surprising fact that was found  
10 for the first time by the inventor of the present invention on the basis of the theory proposed by the inventor. The present invention is completed on the basis of the finding.

**[0017]** Thus, according to the present invention, there is provided a method of detecting a nucleic acid cleavage contained in a single cell, the  
15 method comprising steps (1) through (5) listed below:

- (1) a step of arranging one or more cells on a support having an amino group on the surface thereof;
- (2) a step of covering the cell or cells, whichever is appropriate, with a mixture of purified proteolytic enzyme having a weakly alkaline optimum  
20 pH value and a weakly acidic solution containing a gelling agent;
- (3) a step of solidifying the weakly acidic solution;
- (4) a step of immersing the support carrying thereon the solidified weakly acidic solution into a weakly alkaline solution containing a condensed phosphoric acid compound, a surface active agent and a  
25 reducing agent; and
- (5) a step of separating the nucleic acid derived from the cell or cells, whichever is appropriate, on the support immersed in the weakly alkaline solution by means of pulse-field gel electrophoresis using a solution for electrophoresis containing a condensed phosphoric acid compound.

**[0018]** In another aspect of the present invention, there is provided a method of manufacturing a cell that is free from nucleic acid cleavage, the method comprising steps (1) through (6) listed below:

- (1) a step of arranging one or more cells found in a test sample on a support having an amino group on the surface thereof;
- (2) a step of covering the cell or cells, whichever is appropriate, with a mixture of purified proteolytic enzyme having a weakly alkaline optimum pH value and a weakly acidic solution containing a gelling agent;
- (3) a step of solidifying the weakly acidic solution;
- (4) a step of immersing the support carrying thereon the solidified weakly acidic solution into a weakly alkaline solution containing a condensed phosphoric acid compound, a surface active agent and a reducing agent;
- (5) a step of separating the nucleic acid derived from the cell or cells, whichever is appropriate, on the support immersed in the weakly alkaline solution by means of pulse-field gel electrophoresis using a solution for electrophoresis containing a condensed phosphoric acid compound; and
- (6) a step of determining the cell or cells, whichever is appropriate, in the test sample as cell or cells that are free from nucleic acid cleavage when the mode of separation of the nucleic acid that has been subjected to electrophoresis is substantially similar to the mode of separation of the nucleic acid derived from a normal cell or cells, whichever is appropriate.

**[0019]** For the purpose of the present invention, preferably, the purified proteolytic enzyme having a weakly alkaline optimum pH value is active for dissociation of the peptide bond formed between arginine and arbitrary amino acid and/or the peptide bond between lysine and arbitrary amino acid.

**[0020]** For the purpose of the present invention, preferably, the purified proteolytic enzyme having a weakly alkaline optimum pH value is a

purified trypsin-like enzyme.

**[0021]** For the purpose of the present invention, preferably, the condensed phosphoric acid compound is at least a phosphoric acid compound selected from a group consisting of polyphosphoric acid, hexametaphosphoric acid and salts thereof.

**[0022]** For the purpose of the present invention, preferably, the pulsed field gel electrophoresis is achieved by an electrophoresis apparatus having two or more pairs of electrodes, the two or more pairs of electrodes, whichever is appropriate, being arranged so as to intersect each other at an angle between 45 degrees and 120 degrees.

**[0023]** For the purpose of the present invention, preferably, the pulsed field gel electrophoresis is achieved by executing the first pulsed field gel electrophoresis, turning the support by 60 to 160 degrees around the cell arrangement portion thereof, using the cell arrangement portion as center of revolution, and executing the second pulsed field gel electrophoresis.

**[0024]** For the purpose of the present invention, preferably, the cell or cells are sperm cell or sperm cells, whichever is appropriate.

**[0025]** In still another aspect of the present invention, there is provided a kit for detecting nucleic acid cleavage or for manufacturing a cell with cleavage-free nucleic acid, the kit comprising components (1) through (5) listed below:

- (1) a support having an amino group on the surface thereof;
- (2) purified proteolytic enzyme having a weakly alkaline optimum pH value;
- (3) a weakly acidic solution containing a gelling agent;
- (4) a weakly alkaline solution containing a condensed phosphoric acid compound, a surface active agent and a reducing agent; and
- (5) a solution for electrophoresis containing a condensed phosphoric acid compound.

[0026] Preferably, a kit according to the present invention as defined above further comprises component (6) listed below:

(6) an electrophoresis apparatus having two or more pairs of electrodes, the two or more pairs of electrodes, whichever is appropriate, being arranged so as to intersect each other at an angle between 45 degrees and 120 degrees.

### **Advantages of the Invention**

[0027] Thus, according to the present invention, it is now possible to observe double-stranded DNA composing a chromosome as DNA fibers and, additionally, when the double-stranded DNA in a cell is broken, it is possible to observe the double-stranded DNA as fibrous fragments or as particular fragments depending on the degree of breakage.

[0028] In other words, according to the present invention, it is now possible to observe the number of fibrous fragments produced as the nucleic acid in a cell is cut at one to several points and detect particulate fragments produced as fibrous fragments are shortened as a result of an increase of cutting points and a remarkable progress of fragmentation.

[0029] Thus, the present invention provides an excellent sensitivity of detecting one to several cutting points of the nucleic acid contained in a single cell. Therefore, according to the present invention, it is now possible to detect fibrous DNA fragments in the initial stages of the cleavage that the conventional COMET method cannot detect.

### **Brief Description of the Drawings**

[0030]

FIG. 1A is a photograph of the sperm heads that were observed when purified motile sperms were suspended in a cell lysate. No voltage was applied thereto.

FIG. 1B is a photograph of the sperm heads that were observed  
5 when purified motile sperms were suspended in a cell lysate and purified trypsin was added to the suspension. No voltage was applied thereto.

FIG. 2A is a photograph of the DNA that was observed when purified motile sperms were embedded in agarose and trypsin was added thereto. No voltage was applied to the DNA in FIG. 2A.

10 FIG. 2B is a photograph of the DNA that was observed when ejaculate was embedded in agarose and trypsin was added thereto. No voltage was applied to the DNA in FIG. 2B.

FIG. 2C is a photograph of the DNA that was observed when purified motile sperms were subjected to cytolysis in the presence of  
15 trypsin and then to pulsed field gel electrophoresis.

FIG. 2D is a photograph of the DNA that was observed when ejaculate was subjected to cytolysis in the presence of trypsin and then to pulsed field gel electrophoresis.

FIG. 3A is an enlarged photograph of the DNA fibers that had been  
20 extended by pulsed field gel electrophoresis. No cleavage was observable in the DNA fibers of the DNA.

FIG. 3B is an enlarged photograph of the tip portions of the DNA fibers that had been extended by pulsed field gel electrophoresis. No cleavage was observable in the DNA fibers of the DNA.

25 FIG. 4A is an enlarged photograph of the DNA fibers that had been extended by pulsed field gel electrophoresis. Several filamentous fragments and particular fragments were observed in a front end area of the DNA fibers of the DNA.

FIG. 4B is an enlarged photograph of the DNA fibers that had been

extended by pulsed field gel electrophoresis. Several fibrous fragments and particular fragments were observed in a front end area of the DNA fibers of the DNA.

FIG. 4C is an enlarged photograph of the DNA fibers that had been  
5 extended by pulsed field gel electrophoresis. Several thread-like fragments and particular fragments were observed in a front end area of the DNA fibers of the DNA.

FIG. 5A is a photograph of the DNA that was observed when it was subjected to pulsed field gel electrophoresis after lysing cells of purified  
10 motile sperms without adding trypsin.

FIG. 5B is a photograph of the DNA that was observed when it was subjected to pulsed field gel electrophoresis after lysing cells of ejaculate without adding trypsin.

FIG. 6A is a photograph of the DNA that was observed when it was  
15 subjected to pulsed field gel electrophoresis after alkali-treating purified motile sperms that had been treated with trypsin. The photograph shows that DNA cleavage was artificially induced by the alkali treatment.

FIG. 6B is a photograph of the DNA that was observed when it was subjected to pulsed field gel electrophoresis after alkali-treating purified  
20 motile sperms that had been treated with trypsin. The photograph shows that DNA cleavage was artificially induced by the alkali treatment.

FIG. 6C is a photograph of the DNA that was observed when it was subjected to pulsed field gel electrophoresis after alkali-treating purified motile sperms that had been treated with trypsin. The photograph shows  
25 that DNA cleavage was artificially induced by the alkali treatment.

FIG. 6D is a photograph of the DNA that was observed when it was subjected to pulsed field gel electrophoresis after alkali-treating purified motile sperms that had been treated with trypsin. The photograph shows that DNA cleavage was artificially induced by the alkali treatment.

FIG. 7 is an enlarged photograph of the DNA fibers that had been extended by two-dimensional pulsed field gel electrophoresis. The arrows (→) in FIG. 7 indicate long-chain DNA fragments that cannot be observed by means of one-dimensional pulsed field gel electrophoresis. With the  
5 COMET method, DNA chains are cleaved by a strong alkali treatment to detect nicks.

FIG. 8 is a schematic illustration of the DNA fibers extended by means of one-dimensional pulsed field gel electrophoresis (upper) and those extended by means of two-dimensional pulsed field  
10 electrophoresis (lower).

## **Modes for Carrying Out the Invention**

### **[0031]**

1. Method of Detecting Nucleic Acid Cleavage contained in a single  
15 cell according to the present invention

The present invention relates to a method of detecting nucleic acid cleavage contained in a single cell. In a specific mode of carrying out the present invention, there is provided a method of detecting nucleic acid cleavage contained in a single cell, the method comprising steps (1)  
20 through (5) listed below:

- (1) a step of arranging one or more cells on a support having an amino group on the surface thereof;
- (2) a step of covering the cell or cells, whichever is appropriate, with a mixture of purified proteolytic enzyme having a weakly alkaline optimum  
25 pH value and a weakly acidic solution containing a gelling agent;
- (3) a step of solidifying the weakly acidic solution;
- (4) a step of immersing the support carrying thereon the solidified weakly acidic solution into a weakly alkaline solution containing a

condensed phosphoric acid compound, a surface active agent and a reducing agent; and

(5) a step of separating the nucleic acid derived from the cell or cells, whichever is appropriate, on the support immersed in the weakly alkaline solution by means of pulse-field gel electrophoresis using a solution for electrophoresis containing a condensed phosphoric acid compound.

5 [0032] For the purpose of the present invention, a “cell” may be prepared or isolated from a sample derived from a biological species. An individual prokaryote itself, an individual eukaryote itself or a part of either of them may be used as a sample containing such a cell for the purpose of the present invention. In the instance of a vertebrate (which may be a human being), a sample containing a cell can be obtained from excretions such as feces, urine and sweat, body fluids such as blood, semen, saliva, gastric juice fluid and bile, and biopsy samples.

10 [0033] In the case of using a cell that has not been separated from a tissue, for example, the cell can be separated from the tissue by means of a collagenase treatment.

[0034] Furthermore, exemplar “cells” that can be used for the purpose of the present invention unlimitedly include body cells such as fibroblasts, nerve cells, haemocytes (such as T cells) and germ cells such as sperms, of which germ cells are preferable and sperms are more preferable.

20 [0035] For the purpose of the present invention, “nucleic acid” refers to DNA and RNA contained in a cell (e.g., cell nucleus), of which DNA is preferable and DNA in the cell nucleus is more preferable. DNA in the cell nucleus is synonymous with chromosome DNA and genome DNA.

[0036] For the purpose of the present invention, “nucleic acid cleavage” refers to double-stranded nucleic acid cleavage and single-stranded nucleic acid cleavage.

[0037] Now, “the method of detecting nucleic acid cleavage contained in

a single cell” according to the present invention will be described in detail below.

**[0038]**

(1) A step of arranging one or more cells on a support having an amino group on the surface thereof

In step (1), one or more cells are arranged on a support having an amino group on the surface thereof.

**[0039]** The support on which one or more cells are arranged will then be

arranged in an electrophoresis apparatus at the time of executing pulsed

field gel electrophoresis in step (5). There are no particular limitations to

the support to be used in step (1) so long as it has an amino group on the

surface thereof. Examples of support that can be used in step (1) include

a glass slide having an amino group on the surface thereof. There are no

particular limitations to the method to be used for applying an amino

group onto the surface of the support and techniques that can be used to

apply an amino group onto the surface of the support include techniques of

treating the surface of a support with an amino group-containing

compound, techniques of manufacturing a support so as to orient an amino

group to the surface of the support by using an amino group-containing

compound and other techniques of causing a specific functional group to

express itself on the surface of a support that are known to those who are

skilled in the art.

**[0040]** There are no particular limitations to the method of arranging a

cell or cells on a support for the purpose of the present invention.

Exemplar techniques include a technique of dripping a cell-containing

solution onto the support and centrifugally anchoring the cell onto the

support.

**[0041]**

(2) A step of covering the cell or cells, whichever is appropriate, with a

mixture of purified proteolytic enzyme having a weakly alkaline optimum pH value and a weakly acidic solution containing a gelling agent

In step (2), the cell is or the cells are, whichever is appropriate, covered with a mixture of purified proteolytic enzyme having a weakly alkaline optimum pH value and a weakly acidic solution containing a gelling agent.

**[0042]** For the purpose of the present invention, proteolytic enzyme is an enzyme that can separate the nucleic acid contained in a cell from the nucleoprotein and/or the nucleic matrix by proteolysis.

**[0043]** There are no particular limitations to the proteolytic enzyme to be used in step (2). Examples of such enzymes include those that are active for dissociation of the peptide bond formed between arginine and arbitrary amino acid and/or the peptide bond between lysine and arbitrary amino acid because histone and protamine that are major nucleoproteins are rich of arginine and lysine. Since an ionic bond is formed between the positive electric charge of the basic residues in nucleoproteins and the negative electric charge of the phosphoric acid group in DNA, the enzyme is preferably an enzyme having a function to dissociate the ionic bond. Suitable examples of proteolytic enzymes include trypsin produced from pancreas and derived from any of various animals, trypsin-like proteolytic enzymes having substrate-specific properties that are very similar to trypsin derived from any of various animals and microbes and general purpose proteinase K, of which trypsin is preferable. Trypsin can be made to maintain its purity and activity in acidic conditions, for instance, of pH 2.0 or less.

**[0044]** Purified proteolytic enzyme is employed for the purpose of the present invention. While there are no particular limitations to the degree of purification of the proteolytic enzyme to be used in step (2) so long as it is active for dissociation of nucleoprotein and DNA in a cell, the degree of

purity of the proteolytic enzyme is not less than 50%, preferably not less than 60%, more preferably not less than 70%, further preferably not less than 80%, still more preferably not less than 90% and most preferably not less than 95%. There are no particular limitations to the technique of  
5 purifying the proteolytic enzyme for step (2) and any of the known general purpose proteolytic enzyme purifying techniques that are known to those who are skilled in the art may be used. For example, affinity chromatography that employs a column with one or more substances that have been turned into the solid phase and show a high degree of affinity  
10 for proteolytic enzymes may be used.

**[0045]** For the purpose of the present invention, the proteolytic enzyme to be used in step (2) has a weakly alkaline optimum pH value. The expression of optimum pH value has the ordinary meaning that is known to those who are skilled in the art. For example, it may be the pH value  
15 at which the proteolytic enzyme is relatively most active. The proteolytic enzyme has a weakly alkaline optimum pH value, which is for example between pH 7.0 and pH 12, preferably between pH 7.5 and pH 10.5, more preferably between pH 7.8 and pH 9.0.

**[0046]** Prokaryotes do not have any nucleoprotein and/or any nuclear  
20 matrix but have nucleoid proteins. Therefore, for the purpose of the present invention, proteolytic enzymes to be applied to prokaryotes are those that can separate nucleic acids contained in cells from nucleoid proteins. Examples of such enzymes include trypsin and Proteinase K.

**[0047]** Proteolytic enzymes that can be used for the purpose of the  
25 present invention may be proteins that can be isolated from all the species of living creatures. Additionally, proteolytic enzymes that can be used for the purpose of the present invention may be proteins that are chemically synthesized or biochemically synthesized in cell-free translation systems. Furthermore, proteolytic enzymes that can be used for the purpose of the

present invention may be recombinant proteins produced from transformants into which nucleic acids having a base sequence that encodes proteolytic enzymes have been introduced.

**[0048]** For the purpose of the present invention, not only a single  
5 proteolytic enzyme but also a mixture of a plurality of proteolytic enzymes may be used.

**[0049]** Gelling agents that can be used for the purpose of the present invention include known gelling agents. Examples of known gelling agents include agarose, gelatin, starch, carrageenan, pectin, agaropectin,  
10 polyacrylamide, polyacrylic acid, polyvinyl alcohol, polyvinyl pyrrolidone and trehalose, of which agarose that can form a stable three-dimensional network structure is preferable. This is because such agarose can form stable three-dimensional structure without using factors that can adversely affect DNA such as oxidizing agents and ultraviolet rays.

15 **[0050]** The concentration of the agarose to be used is set to a low concentration level by considering the length of DNA in the cell nucleus. For example, the agarose concentration (final concentration) is between 0.1 and 1.0 w/v%, preferably between 0.1 and 0.6 w/v%, more preferably between 0.2 and 0.5 w/v%, most preferably between 0.2 and 0.3 w/v%.

20 **[0051]** For the purpose of the present invention, a solubilizing agent may be added to the weakly acidic solution in order to solubilize the cell membrane.

**[0052]** Examples of solubilizing agents that can be used for this step include surface active agents. The use of a surface active agent that  
25 mildly acts on the proteolytic enzyme is preferable. Examples of such surface active agents include digitonin, saponin, Triton X100, Triton X114, Tween 20 and Tween 80, all of which are commercially available.

**[0053]** The pH range of the weakly acidic solution is, for example between pH 3.0 and 7.0, preferably between pH 4.0 and 6.0. There are no

particular limitations to the method of adjusting the weak acidity of the weakly acidic solution. For example, the weak acidity of the solution can be adjusted by making the solution contain an acid or a buffer solution of a certain concentration whose acidity has been adjusted to a weakly acidic level.

**[0054]** All the remaining conditions of step (2) such as the concentration of the solution, the processing temperature and the processing time may vary depending on the type of the cell and the contents such as the proteolytic enzyme, the gelling agent and the solubilizing agent.

However, those who are skilled in the art can appropriately set those conditions. There are no particular limitations to the technique of covering the cell or cells arranged on the support with a mixture of a purified proteolytic enzyme having a weakly alkaline optimum pH value and a weakly acidic solution containing a gelling agent. For example, a technique of dripping the mixture onto the part of the support that has been smeared with the cell or cells, whichever is appropriate, may be employed.

**[0055]** Furthermore, in step (2), the pH value is adjusted so as not to become not less than 10, preferably so as to become neutral or weakly acidic. This is because nucleic acid fragmentation is induced by an alkali treatment as will be described hereinafter. The operation of step (2) is preferably executed at a relatively high temperature such as at a temperature not lower than 30°C by which the gelling agent can be held in a molten state. However, if the temperature is too high, there will arise a probability that the enzyme is deactivated and the nucleic acid is denatured.

**[0056]**

(3) A step of solidifying the weakly acidic solution of Step (2)

In step (3), the weakly acidic solution of step (2) is solidified.

**[0057]** For example, the weakly acidic solution can be solidified by adding a proteolytic enzyme and a gelling agent to the solution that has been heated and the temperature of the obtained heated solution is lowered to room temperature (e.g., a temperature between 10 and 30°C.

5 When chemical polymerization is required, a polymerization initiator may be added depending on the characteristics of the gelling agent.

**[0058]** As a result of the operations of steps (2) and (3), the nucleic acids such as the DNA in the cell nucleus are embedded into the gel. The operation of step (3) is executed in conditions that inactivate the  
10 proteolytic enzyme including, for example, a weakly acidic condition.

**[0059]**

(4) A step of immersing the support carrying thereon the solidified weakly acidic solution that was solidified in step (3) into a weakly alkaline solution containing a condensed phosphoric acid compound, a surface  
15 active agent and a reducing agent

In step (4), the support carrying thereon the weakly acidic solution that was solidified in step (3) is immersed into a weakly alkaline solution, which is a cell lysate.

**[0060]** There are no particular limitations to the composition of the  
20 weakly alkaline solution so long as it contains a condensed phosphoric acid compound, a surface active agent and a reducing agent. For example, the weakly alkaline solution may further contain a buffer and one or more other components. A condensed phosphoric acid compound refers to a newly produced molecule ( $H_{n+2}P_nO_{3n+1}$  (where n is an integer not smaller  
25 than 1)) obtained as two or more phosphoric acid molecules react with each other and the water molecules are eliminated therefrom or a salt thereof.

**[0061]** The reason for adding the condensed phosphoric acid compound to the cell lysate will be described below. Namely, the structure of DNA is such that the DNA backbone is formed by repetitive phosphodiester bonds

of deoxyribose (pentose) and phosphoric acid and nucleobases are attached to the DNA backbone to produce a helical configuration. The phosphate group in a phosphodiester bond has a strong negative charge. On the other hand, arginine and lysine that form the nucleoprotein has a positive charge so that nucleoprotein and phosphoric acid are electrostatically coupled. The condensed phosphoric acid compound can efficiently dissociate the DNA as it is competitively and electrostatically coupled to the nucleoprotein. As the condensed phosphoric acid compound can specifically and competitively dissociate the binding of the nucleoprotein and the DNA in this way, the concentration thereof may be held to a low level, for example, of not higher than 10 mM.

**[0062]** Conventionally, one or more highly concentrated salts (such as NaCl of not less than 2M) are employed for solubilization and dissociation of nucleoprotein and DNA. The inventor's attention was focused on the fact that a slight amount of condensed phosphoric acid compounds such as hexametaphosphoric acid and polyphosphoric acid can dissociate DNA-nucleoprotein complexes and adopted this fact for the purpose of verification of DNA fragmentation for the first time. Suitable examples of condensed phosphoric acid compounds include hexametaphosphoric acid, salts thereof, polyphosphoric acid and salts thereof.

**[0063]** Meanwhile, it may be conceivable to turn steps (2) through (4) of the present invention as described above into a single step of solidifying the weakly acidic solution containing a gelling agent in a state of not allowing the weakly acidic solution to contain any proteolytic enzyme and immersing the support in the cell lysate containing a proteolytic enzyme. However, it is difficult to treat the cells that are embedded into the gel with the proteolytic enzyme that is added thereafter. This is because, after the solidification of the gel, the reagents with low molecular weight (selected from DTT, salts, etc.) can be diffused with ease in the gel network

structure but it is difficult for a proteolytic enzyme having a molecular weight of tens of thousands or more to be diffused in the gel and get to near and around the cell or cells.

**[0064]** Steps (2) through (4) are devised to overcome the above identified difficulty. In step (3), the weakly acidic solution containing the proteolytic enzyme and the gelling agent of step (2) is solidified. As a result of executing the operations of these steps, the proteolytic enzyme is brought into a state of being uniformly diffused into the solidified gel.

**[0065]** What is important here is that the operations of steps (2) and (3) are executed in a condition where the proteolytic enzyme is kept inactive, whereas the operation of step (4) is executed in a condition where the proteolytic enzyme is activated. This is because if the cell is or the cells are lysed by the proteolytic enzyme before the gel is solidified, the DNA is diffused in the solution to make it difficult to determine the DNA

fragments derived from a single cell. Therefore, since the proteolytic enzyme that is employed for the purpose of the present invention has a weakly alkaline optimum pH value, the operation of step (2) is executed in a condition that shows a pH value of other than weakly alkaline, for example in a weakly acidic condition, whereas the operation of step (3) is executed in a weakly alkaline condition. The expression that “the proteolytic enzyme is active” refers to that the proteolytic enzyme decomposes proteins. The expression that “the proteolytic enzyme is inactive”, on the other hand, refers to that the proteolytic enzyme substantially does not digest any proteins.

**[0066]** For example, the proteolytic activity of bovine pancreatic trypsin is pH dependent and is active when pH value is between about 6 and 10. On the basis of this fact, it is possible to execute the operations of steps (2) and (3) in a condition where the proteolytic enzyme is made inactive and execute the operation of step (4) in a condition where the proteolytic

enzyme is made active by selecting a weakly acidic condition where the pH value is less than 6 for steps (2) and (3) and selecting a condition where the pH value is between about 6 and 10 for step (4).

**[0067]** As a more specific example, trypsin is inactive in a gel obtained by solidifying a pH 4.7 solution containing trypsin and agarose. Then, the agarose is immersed in a cytolytic solution at pH 8.1, the trypsin is activated to start a proteolytic reaction.

**[0068]** Since the reaction of the enzyme is influenced by the temperature, the enzyme concentration, the reaction time, the presence or absence of an enzyme inhibitor and other factors in addition to the pH value, it is possible to appropriately select the conditions in which the operations of steps (2) through (4) are executed so as to produce a situation where “the proteolytic enzyme is active” or a situation where “the proteolytic enzyme is inactive” in accordance with the properties of the proteolytic enzyme.

**[0069]**

(5) A step of separating the nucleic acid derived from the cell or cells on the support that is or are, whichever is appropriate, treated in step (4) by means of pulse-field gel electrophoresis using a solution for electrophoresis containing a condensed phosphoric acid compound

In step (5), the nucleic acid derived from the cell or cells on the support treated in step (4) is isolated by means of pulse-field gel electrophoresis using a solution for electrophoresis containing a condensed phosphoric acid compound.

**[0070]** The operation of pulsed field gel electrophoresis is executed by shifting the direction of the electric field at regular time intervals by means of a plurality of pairs of electrodes. With the pulsed field gel electrophoresis, as the direction of the electric field is shifted at regular time intervals, morphological changes of the nucleic acid molecule is

induced and, as a result, the huge nucleic acid molecule can be moved so as to slip through the network of the gel in a subtle manner and consequently become isolated.

**[0071]** According to the present invention, prior to executing the operation of electrophoresis, the nucleic acid is located at a position or positions where the cell is or the cells are, whichever is appropriate, embedded in the gel. As a result of executing the operation of electrophoresis by means of a pulsed field gel electrophoresis technique, the nucleic acid that has been entangled in the gel can be disentangled and extended from the original position (the position where the nucleic acid is located prior to executing the operation of electrophoresis).

**[0072]** The operation of electrophoresis can be executed in a manner that is known to those who are skilled in the art. For instance, the electrophoresis tank to be used for the pulsed field gel electrophoresis may be made to comprise two pairs of electrodes that intersect each other at an angle between 40 and 120 degrees, more specifically at an angle selected from a group of angles including 40 degrees, 45 degrees, 50 degrees, 60 degrees, 70 degrees, 90 degrees, 100 degrees, 110 degrees and 120 degrees or three or more pairs of electrodes located within the above angular range. Then, the support treated in step (4) is placed such that the cell arrangement position is located at the intersection of the pairs of electrodes in the pulsed field gel electrophoresis system and the pairs of electrodes are electrically alternately energized for electrophoresis.

**[0073]** Additionally, with regard to electrophoresis by pulsed field gel electrophoresis, an operation of electrophoresis can be repeated twice or more than twice by rotating the support and shifting the direction of electrophoresis. For example, the objective of pulsed field gel electrophoresis of step (5) can be achieved by executing the first operation of pulsed field gel electrophoresis, subsequently turning the support by a

certain angle around the cell arrangement portion thereof so as to make the cell arrangement portion operate as center of revolution and executing the second pulsed field gel electrophoresis. Thus, by executing an operation of electrophoresis twice or more than twice, shifting the direction of electrophoresis, it is possible to detect long nucleic acid fragments entangled in gel that cannot be isolated by the first electrophoresis.

**[0074]** The differences between the results of one-dimensional pulsed field gel electrophoresis and those of two-dimensional pulsed field gel

electrophoresis will be described below by referring to FIG. 8 that schematically illustrates those results. In FIG. 8, the upper part schematically illustrates the results of “one-dimensional electrophoresis” (upper). After the operation of one-dimensional pulsed field gel

electrophoresis, long chain DNA extending from the original position , fibrous DNA fragments separated from the front end part of the long chain DNA (a), shorter DNA chains separated from the front end part of the fibrous DNA fragments (b) and particulate fragments (c) were observed.

In contract, after the two-dimensional pulsed field gel electrophoresis, as schematically shown in the lower part of FIG. 8 illustrating the results of

“two-dimensional electrophoresis” (lower), long chain DNA that had not been extended by the first operation of electrophoresis was extended and very long DNA fragments that could not be separated by one-dimensional pulsed field gel electrophoresis because they were entangled with the DNA chains that were extending from the original position were detected (d).

Additionally, the DNA fragments and particles located in blocks a, b, c and d are forced to migrate in different directions so that consequently DNA fragments of various different lengths that were running substantially in parallel in the longitudinal direction became observable. Therefore, with two-dimensional pulsed field gel electrophoresis, when no DNA fragment

is detected in the blocks of a, b, c and d where DNA fragments presumably has been forced to migrate, DNA in the cell nucleus of the cell can be determined to have not been cut.

**[0075]** While there are no particular limitations to the angle by which the support is turned after the first operation of pulsed field gel electrophoresis in two-dimensional pulsed field gel electrophoresis so long as the angle allows to detect long chain DNA fragments, which cannot be detected by one-dimensional pulsed field gel electrophoresis, without being hindered by the DNA chains extending from the original position, the support is typically turned by an angle between 60 and 160 degrees around the cell arrangement portion thereof so as to make the cell arrangement portion operate as center of revolution. More specifically, the angle is selected from a group of angles consisting of 60 degrees, 70 degrees, 80 degrees, 90 degrees, 100 degrees, 110 degrees, 120 degrees, 130 degrees, 140 degrees, 150 degrees and 160 degrees. When the angle is 140 degrees, the DNA fragments and particles in the blocks of a, b, c and d can clearly be detected as schematically illustrated in the lower part of FIG. 8.

**[0076]** With the conventional COMET method, the pattern according to which particulate fragments are driven to migrate from the original position by electrophoresis is analyzed. Since the conventional COMET method does not involve digestion of nucleoprotein by means of proteolytic enzyme and normally employs only a single set of electrodes for electrophoresis, it cannot isolate the nucleic acid entangled in the gel from the nucleoprotein, disentangle the nucleic acid and then extend the nucleic acid.

**[0077]** There are no particular limitations to the composition of the electrophoretic solution to be used in step (5) so long as it contains a condensed phosphoric acid compound. For example, some of all of the

components of ordinary electrophoretic buffer solution may be added to it. As described above for step (4), it is possible to prevent re-association of nucleoprotein digests that are on the way of electrophoretic migration and DNA by adding a condensed phosphoric acid compound. Samples

5 obtained by eliminating protein have hitherto been employed for electrophoresis of DNA in the prior art DNA researches. However, even after treating with a proteolytic enzyme, basic peptides derived from the decomposed nucleoprotein coexist in the electrophoresis system and hence these basic peptides are bound to the DNA again to remove the negative  
10 electric charge of the DNA and hinder the progress of electrophoresis.

For this reason, the electrophoretic solution is made to contain a condensed phosphoric acid compound for the purpose of the present invention. For the same reason, the electrophoretic solution is preferably a weakly alkaline solution wherein the proteolytic enzyme is active in  
15 order to further decompose the basic peptides that have been released from the nucleoprotein.

**[0078]** The condensed phosphoric acid compound contained in the weakly alkaline solution of step (4) and the condensed phosphoric acid compound contained in the electrophoretic solution of step (5) may be same  
20 with each other or different from each other. Furthermore, each of the weakly alkaline solution of step (4) and the electrophoretic solution of step (5) may contain two or more condensed phosphoric acid compounds. Note, however, when conducting the operation of two-dimensional pulsed field gel electrophoresis, the condensed phosphoric acid compound contained in  
25 the weakly alkaline solution is preferably a hexamethaphosphate and the condensed phosphoric acid compound contained in the electrophoretic solution is preferably a polyphosphate or a hexamethaphosphate.

**[0079]** As for the conditions in which the operation of electrophoresis is conducted including the electrophoresis voltage to be used for pulsed field

gel electrophoresis, the timing of switching the pairs of electrodes and the duration of electrophoresis, there are no particular limitations to the conditions in the case of one-dimensional pulsed field gel electrophoresis. However, in the case of two-dimensional pulsed field gel electrophoresis, the two pairs of electrodes are alternately electrically energized with a potential difference between 1.0 and 2.0 V/cm by every 3 to 5 seconds for a total of 5 to 10 minutes for the first round of electrophoresis and then the two pairs of electrodes are alternately electrically energized also with a potential difference between 1.0 and 2.0 V/cm by every 3 to 5 seconds for a total of 5 to 10 minutes for the second round of electrophoresis.

**[0080]** The nucleic acid that was extended by electrophoresis in step (5) can be observed through a fluorescence microscope when it is stained typically with a nucleic acid staining dye. There are no particular limitations to the nucleic acid staining pigment to be used for the purpose of the present invention so long as it emits fluorescence, examples of nucleic acid staining pigments that can be used for the purpose of the present invention include SYBR Gold (trade name, available from Life Technologies). Then, the nucleic acid cleavage contained in a single cell is visualized so that it can typically be observed through a microscope.

**[0081]** According to the present invention, double-stranded DNA that constitutes chromosome can be observed as DNA fibers (refer to, for example, FIG. 7 as well as FIGS. 3A and 3B). As double-stranded DNA starts to be broken, cleavage takes place at several positions so that several fibrous fragments are produced due to the cleavage (refer to, for example, FIGS. 4A and 4B). As the cleavage progresses, the produced fragments increase in number and become shorter. As the cleavage progresses further, particulate fragments appear (refer to, for example, FIG. 4C). As the fragmentation progresses furthermore, the quantity of the nucleic acid found at the original position (the position where the

nucleic acid was located prior to the electrophoresis) decreases and hence eventually a reduced size of the nucleic acid at the original position is observed through a microscope.

**[0082]** Thus, according to the present invention, it is possible to observe and detect the cleavage, if any, at one to several positions of the double-stranded DNA contained in a single cell and an increase in the number and the reduction of length of the thread-like fragments that are cut out as well as particular fragments.

**[0083]** The present invention is characterized by (i) executing a proteolytic enzyme treatment, (ii) not conducting any alkali treatment and (iii) using a pulsed field gel electrophoresis technique among others.

**[0084]** To the contrary, the conventional COMET method is characterized by (i) not executing any treatment of proteolytic enzyme, (ii) executing an alkali treatment and (iii) executing an operation of electrophoresis by using only a single set of electrodes. Thus, the conventional COMET method cannot provide correct results for the reasons listed below.

**[0085]** Namely, (i) since it does not execute any proteolytic enzyme treatment, it produces false negativity (refer to, for example, FIGS. 5A and 5B), (ii) when it executes an alkali treatment, DNA is artificially broken to give rise to false positivity (refer to, for example, FIGS. 6A through 6D) and (iii) with an operation of electrophoresis using a single set of electrodes, it is not possible to disentangle the nucleic acid entangled in gel and extend the nucleic acid.

**[0086]** As the conventional COMET method gives rise to both false negativity and false positivity, it is now clear that any correct results cannot be obtained by the method.

**[0087]**

2. Method of manufacturing a cell that is free from nucleic acid

cleavage

The present invention also relates to a method of manufacturing a cell that is free from nucleic acid cleavage. In a specific mode of the present invention, there is provided a method of manufacturing a cell that is free from nucleic acid cleavage, the method comprising steps (1) through (6) listed below. For the purpose of the present invention, the expression of "manufacturing a cell" refers to acquiring a specific cell. As far as the present invention is concerned, the expression of "manufacturing a cell" refers to obtaining a specific cell and includes the expressions of "preparing a cell", "isolating a cell" and "extracting a cell."

The method comprises:

- 1) a step of arranging one or more cells found in a test sample on a support having an amino group on the surface thereof;
- (2) a step of covering the cell or cells, whichever is appropriate, with a mixture of purified proteolytic enzyme having a weakly alkaline optimum pH value and a weakly acidic solution containing a gelling agent;
- (3) a step of solidifying the weakly acidic solution;
- (4) a step of immersing the support carrying thereon the solidified weakly acidic solution into a weakly alkaline solution containing a condensed phosphoric acid compound, a surface active agent and a reducing agent;
- (5) a step of separating the nucleic acid derived from the cell or cells, whichever is appropriate, on the support immersed in the weakly alkaline solution by means of pulse-field gel electrophoresis using a solution for electrophoresis containing a condensed phosphoric acid compound; and
- (6) a step of determining the cell or cells, whichever is appropriate, in the test sample as being free from nucleic acid cleavage when the mode of separation of the nucleic acid that has been subjected to electrophoresis is substantially similar to the mode of separation of the nucleic acid derived

from a normal cell or cells, whichever is appropriate.

[0088] As far as the present invention is concerned, there are no particular limitations to the test sample for the above defined method so long as it contains a cell or cells and the cell or cells, whichever is appropriate, may be derived from a living body or originating from nature. For example, if it is desired to manufacture sperm that is free from nucleic acid cleavage, semen may be used as test sample.

5 [0089] The operations of steps (1) through (5) can be executed just same as those of steps (1) through (5) of the above described method of detecting a DNA cleavage contained in a single cell. Therefore, step (6) of the method under consideration will be described in detail below.

[0090]

(6) A step of determining the cell or cells, whichever is appropriate, in the test sample as being free from nucleic acid cleavage when the mode of separation of the nucleic acid that has been subjected to electrophoresis is substantially similar to the mode of separation of the nucleic acid derived from a normal cell or cells, whichever is appropriate.

15 Thus, in step (6) when the mode of separation of the nucleic acid that has been subjected to electrophoresis is substantially similar to the mode of separation of the nucleic acid derived from a normal cell or cells, the cell or cells, whichever is appropriate, in the test sample is determined as a cell that is or as cells that are, whichever is appropriate, free from nucleic acid cleavage.

[0091] For the purpose of the present invention, the mode of separation of nucleic acid refers to the mode of presence of DNA fragments and particles in the blocks of a, b, c and d shown in FIG. 8 schematically illustrating two-dimensional pulsed field gel electrophoresis (in the lower part). If the mode of presence of DNA fragments and particles a, b, c and d is similar to that of DNA fragments and particles in the blocks of a, b, c

and d of nucleic acid derived from a normal cell, the cell or cells in the test sample can be determined as a cell that is or as cells that are, whichever is appropriate, free from nucleic acid cleavage. The determination may be either quantitative or qualitative determination. Examples of qualitative determination and quantitative determination will be shown below. Note, however, the examples of determination shown below can be realized by means of a program, by an automatic means or by a mechanical means without relying on determination by those who are skilled in the art as may be clear from the criteria of determination.

10 **[0092]** As an exemplar qualitative determination, the number of the DNA fragments and particles in all or in each of the blocks a, b, c and d that have been separated from the nucleic acid subjected to electrophoresis in step (5) is statistically compared with the corresponding number of the DNA fragments and particles separated from nucleic acid derived from a normal cell and if the difference is statistically insignificant, the nucleic acid of the cell in the test sample can be determined to be free from cleavage.

20 **[0093]** As an exemplar quantitative determination, the possibility (%) that the nucleic acid of the cell in the test sample is free from cleavage is predefined for each range of correlation between the number of the DNA fragments and particles in all or in each of the blocks a, b, c and d that have been separated from the nucleic acid subjected to electrophoresis in step (5) and the corresponding number of the DNA fragments and particles separated from nucleic acid derived from a normal cell and the possibility (%) is determined on the basis of the mode of separation of the nucleic acid of the cell in the test sample and that of the nucleic acid of a normal cell. If the correlation is in the range between A and B, the possibility that the nucleic acid of the cell in the test sample is determined to be free from cleavage is not greater than 10%: if the correlation is in the range between

B and C, the possibility that the nucleic acid of the cell in the test sample is determined to be free from cleavage is between 10% and 30%; if the correlation is in the range between C and D, the possibility that the nucleic acid of the cell in the test sample is determined to be free from cleavage is between 30% and 50%; if the correlation is in the range between D and E, the possibility that the nucleic acid of the cell in the test sample is determined to be free from cleavage is between 50% and 70%; if the correlation is in the range between E and F, the possibility that the nucleic acid of the cell in the test sample is determined to be free from cleavage is between 70% and 90%; if the correlation exceed F, the nucleic acid of the cell in the test sample is determined to be free from cleavage.

**[0094]** Note, however, if the nucleic acid of a normal cell is subjected to electrophoresis of step (5), there exists a probability that DNA fragments and particles in the blocks of a, b, c and d cannot be detected. For this reason, the mode of separation of nucleic acid derived from a normal cell may be predefined as a mode of separation where the number of DNA fragments and particles in the blocks a, b, c and/or d is equal to nil in step (6).

**[0095]** A normal cell refers to a cell that is recognized to be free from abnormality in terms of phenotype such as morphological appearance and proliferation, and genotype such as expression of a specific gene. For the purpose of the present invention, a normal cell may simply be a control cell selected by a test subject.

**[0096]**

3. Kit according to the present invention

The present invention also provides a kit for executing either one or both of the methods of the present invention. In a specific mode of carrying out the present invention, there is provided a kit for detecting nucleic acid cleavage or for manufacturing a cell that is free from nucleic

acid cleavage, the kit comprising components (1) through (5) listed below:

- (1) a support having an amino group on the surface thereof;
- (2) purified proteolytic enzyme having a weakly alkaline optimum pH value;
- 5 (3) a weakly acidic solution containing a gelling agent;
- (4) a weakly alkaline solution containing a condensed phosphoric acid compound, a surface active agent and a reducing agent; and
- (5) a solution for electrophoresis containing a condensed phosphoric acid compound.

10 **[0097]** The components (1) through (5) of a kit according to the present invention are those that have already been described above by referring to the methods of the present invention. A kit according to the present invention may further comprise component (6) listed below:

- (6) an electrophoresis apparatus having two or more pairs of
- 15 electrodes, the two or more pairs of electrodes, whichever is appropriate, being arranged so as to intersect each other at an angle between 45 degrees and 120 degrees.

## **Examples**

20 **[0098]** Now, the present invention will be described further by way of specific examples. Note, however, that the examples by no means limit the scope of the present invention.

### **[0099]**

#### 1. Purification of trypsin

25 Usually, commercially available bovine pancreatic trypsin preparations contain active trypsin only by not more than 20% because of self-digestion and other reasons. Additionally, since commercially available bovine pancreatic trypsin preparations are derived from

pancreas, they are contaminated by DNase, which is an impurity. The inventor of the present invention conducted an experiment of purifying trypsin by means of affinity chromatography with an immobilized Lima bean trypsin inhibitor (LBTI). A column filled with an LBTI-immobilized adsorbent was equilibrated with 20mM of Tris-HCL (pH 8.3) and 1.0 mM of CaCl<sub>2</sub> in advance. Trypsin powder was dissolved in the solution and added to the column. Then, the non-adsorbed impurities were washed out by means of an equilibrating solution with an addition of 0.2M of NaCl until the absorption at 280 nm of the eluate became not greater than 0.01 as observed by a spectrophotometer. The adsorbed trypsin was eluted with 2.0% acetic acid solution (pH 1.8). The obtained preparation was diluted with 2.0% acetic acid solution until the trypsin concentration became 20 µg/ml and subsequently cryopreserved.

#### [0100]

### 2. Pulsed field gel electrophoresis (1)

#### (1) Method

The Percoll density gradient centrifugation method and the swim up method were combined in order to purify the motile sperm obtained from human semen. A glass slide was smeared with  $2 \times 10^4$  cells of the purified motile sperm within an area of  $7 \times 7$  mm. The surface of the glass slide had been labeled with an amino group.

**[0101]** Dissolved 0.56% agarose (0.1M of sodium acetate (pH 4.7), 0.05% of TritonX-100) was filtered through a 0.22 µm Millipore filter and 540 µl thereof and 60 µl of purified trypsin were mixed with each other at 40°C. The prepared agarose was embedded onto the glass slide around the sperm-smeared position, which took the role of center, within an area of  $2.5 \times 2.5$  cm to a thickness of 50 µm and solidified at low temperature for 30 minutes. The solidified gel was immersed in a cell lysate (30 mM of Tris-polyphosphate, 8.2 mM of Na hexametaphosphate, 0.05% of Triton

X-100, 5.0 mM of dithiothreitol, pH 8.1) and allowed to react at 37°C for 30 minutes.

**[0102]** An electrophoresis tank equipped with two pairs of electrodes that intersect each other at an angle of 90° was employed for pulsed field gel electrophoresis. 30 mM of Tris-polyphosphate (pH 8.1) was used as migration liquid solution. The glass slide was placed such that the site smeared with cells was located at the intersection of the two pairs of electrodes and the two pairs of electrodes were alternately electrically energized with a potential difference of 1.5 V/cm by every 3 seconds so as to make the electrophoresis run for a total of 7 minutes.

**[0103]**

## (2) Results

When purified motile sperm was suspended in a cell lysate to which SYBR Gold (trade name, available from Life Technologies) had been added without causing cells to be digested by proteolytic enzyme, the sperm heads were swollen in few minutes but DNA fibers remained in the swollen sperm heads (FIG. 1A). On the other hand, when purified trypsin (20 µg/ml) was added to the suspension, what was observed there was that DNA fibers diffused around the sperm heads in tens of several seconds (FIG. 1B). Since DNA fibers freely diffuse in a suspension in this way, it is difficult to identify the DNA fibers and the fragments thereof derived from a single cell.

**[0104]** In view of this fact, purified motile sperm and ejaculate were embedded in agarose and trypsin was added thereto for cytolysis, the purified motile sperm got into a state where the ends of entangled DNA fibers protruded into the surrounding area (FIG. 2A). On the other hand, the ejaculate showed various morphological appearances including a morphological appearance similar to that of purified motile sperm and a morphological appearance where particulate fragments concentrically

diffused into the surrounding area (FIG. 2B). They freely diffused in the agarose network structure without being applied with a voltage. On the basis of the particulate fragments that were observed in the ejaculate, it will be safe to say that there is a probability that the sperm in the semen is sperm where DNA fragmentation had already progressed.

[0105] Then, the sperm cells that had been lysed in the presence of trypsin were subjected to electrophoresis by pulsed field gel electrophoresis. In the case of the purified motile sperm, tens of several uniform DNA fibers extended from the original position (where the nucleic acid was located prior to the electrophoresis) (FIG. 2C). On the other hand, in the case of the ejaculate, extremely diverse electrophoretic profiles were observed (FIG. 2D). In the case of the ejaculate, extended DNA fibers that were observed in the case where purified motile sperm was used and additionally those from the tip portions of which thread-like fragments had been separated were found. The number and the length of the thread-like fragments varied remarkably from sperm cell to sperm cell.

[0106] FIG. 3A is an enlarged photograph of the DNA fibers that had been extended by pulsed field gel electrophoresis. FIG. 3B is an enlarged photograph of the tip portions of the DNA fibers.

[0107] When the DNA fibers that had been made to migrate were examined through a microscope with a high magnification, it was observed that DNA fibers had extended zigzag in the agarose network structure as a result of application of a voltage for the pulsed field gel electrophoresis.

[0108] The cells of the purified motile sperm were divided into a group where only DNA fibers were observed (FIGS. 3A and 3B) and a group where both DNA fibers and several thread-like fragments at the front ends of the DNA fibers were recognizable (FIGS. 4A through 4C). More specifically, the inventor of the present invention found a surprising fact

that the cells of purified motile sperm include those that are in the initial stages of fragmentation. A method of detecting nucleic acid cleavage in a single cell according to the present invention provides an excellent sensitivity of detecting even one to several sites of DNA cleavage contained in a single cell and can detect DNA cleavage in the initial stages thereof.

[0109] Thus, the inventor of the present invention conducted an experiment as described below in order to prove that even purified motile sperm can contain cleavage in the initial stages thereof.

[0110] Semen ( $n = 8$ ;  $\text{vol} = 2.5 \pm 0.62 \text{ ml}$ ;  $\text{concentration} = 58 \pm 26 \times 10^6$  sperm cells/ml;  $\text{motility} = 59 \pm 17\%$ ) was treated in the above described manner to obtain purified motile sperm ( $\text{vol} = 1.0 \text{ ml}$ ;  $\text{concentration} = 5.3 \pm 2.7 \times 10^6$  sperm cells/ml;  $\text{motility} = 94 \pm 2.1\%$ ) in order to count the number of fragments of the purified motile sperm. Subsequently, the obtained purified motile sperm was subjected to electrophoresis by pulsed field gel electrophoresis in the above described manner. The percentage of the purified motile sperm cells from which no fragments were detected was between 58.9% and 87.2% ( $77.7 \pm 9.47\%$ ). Of all the specimens, the percentage of the purified motile sperm cells from which no fragments were detected was significantly lower if compared with the motility. This fact indicates that even purified motile sperm includes fragments thereof that are in the initial stages of fragmentation.

[0111]

### (3) Comparative Examples

The preceding description on "comparison between the present invention and the conventional COMET method" was verified by Comparative Examples shown below.

[0112]

#### (i) Comparative Example for an instance of not using trypsin

When cytotoxicity is conducted without adding trypsin, the obtained

electrophoretic profile in the pulsed field was quite different from the electrophoretic profile obtained by cytolysis with the addition of trypsin. More specifically, in the case of purified motile sperm, the DNA fibers did not extend at all from the original position (the position where the nucleic acid was located prior to the electrophoresis) (FIG. 5A). In the case of ejaculate, only a small amount of particulate fragments were driven to migrate but no DNA fibers could not be observed (FIG. 5B). These facts indicate that, when trypsin is added, DNA gets into a state of being held in the nuclear matrix to make it impossible to observe both DNA fibers and thread-like fragments cut out from them by means of electrophoresis.

#### [0113]

(ii) Comparative Example for an instance of executing an alkali treatment

The influence of an alkali treatment on DNA was observed.

Purified motile sperm that had been subjected to cytolysis with the addition of trypsin was immersed in 0.05 mol/L and 0.1 mol/L NaOH solutions at room temperature for 10 minutes, subsequently washed with water and subjected to pulsed field gel electrophoresis. In the case of the 0.05 mol/L NaOH solution, it was found that only particulate fragments of DNA had been driven to migrate and no DNA fibers were observable (FIG. 6A). In the case of 0.1 mol/L NaOH solution, on the other hand, it was found that the nucleic acid at the original position (the position where the nucleic acid was located prior to the electrophoresis) had shrunk and the fragmentation had progressed further (FIG. 6B).

[0114] Then, after the pulsed field gel electrophoresis, the purified motile sperm was immersed in a 0.1 mol/L NaOH solution to find that the extended DNA fibers had completely been fragmented (FIG. 6C). On the other hand, in the case of the purified motile sperm that had been subjected to cytolysis by using 0.1 ml/L NaOH solution without adding any

trypsin, it was found that only a small amount of particulate fragments were driven to migrate (FIG. 6D).

[0115] Therefore, while the COMET method that involves an alkali treatment entails a high possibility of giving rise to false positivity for DNA fragmentation, the method of the present invention that does not involve any alkali treatment, DNA cleavage due to an alkali treatment never arises and hence expectedly would not give rise to the problem of false positivity.

[0116]

10 (4) Cells other than sperm cells

While the above-described results were obtained by the use of sperm cells, nucleic acid cleavage contained in a single cell can be detected if the cell is a cell other than a sperm cell as in the case where the cell is sperm.

15 [0117] The present invention is characterized among others in that the DNA in a cell nucleus can be separated from the nucleoprotein and the nuclear matrix by adding proteolytic enzyme into gel and conducting a proteolytic enzyme treatment. In a cell other than a sperm cell, the DNA in the cell nucleus is rigidly anchored to the nucleoprotein and/or the nuclear matrix. Therefore, the nucleic acid cleavage, if any, contained in a single cell can be detected by the method of the present invention if the cell is a cell other than a sperm cell as in the case where the cell is sperm.

3. Pulsed field gel electrophoresis (2)

(1) Method

25 The Percoll density gradient centrifugation method and the swim up method were combined in order to purify the motile sperm obtained from human semen. A glass slide was smeared with  $2 \times 10^4$  cells of purified motile sperm within an area of  $7 \times 7$  mm. The surface of the glass slide was labeled with an amino group.

**[0118]** Dissolved 0.3% agarose (0.1M sodium acetate (pH 4.7), 0.05% Triton X-100) was filtered through a 0.22  $\mu\text{m}$  Millipore filter and 540  $\mu\text{l}$  thereof and 60 $\mu\text{l}$  of purified trypsin were mixed with each other at 40°C. The prepared agarose was embedded in the glass slide to a thickness of 50  $\mu\text{m}$  within an area of 2.5×2.5 cm, the center of the embedded area being the position smeared with sperm, and solidified at low temperature for 30 minutes. The solidified gel was then immersed in a cell lysate (30 mM of Tris-HCL, 1.0 mM of sodium hexametaphosphate, 0.05% Triton X-100, 5.0 mM of dithiothreitol, pH 8.1) and caused to react at 37°C for 30 minutes.

**[0119]** An electrophoresis tank having two pairs of electrodes that were arranged so as to intersect each other at an angle of 60 degrees was employed for the pulsed field gel electrophoresis. As migration liquid, a solution containing 30 mM of Tris-acetate, 1.0mM of Na hexametaphosphate (pH 8.1) or 1.0 mM of Tris-acetate and 30 mM of Tris-polyphosphate (pH 8.1) was used. The glass slide was placed such that the position smeared with the cells is located at the intersection of the two pairs of electrodes and the two pairs of electrodes were alternately electrically energized with a potential difference of 1.5 V/cm by every 3 seconds so as to make electrophoretic migration to take place for a total of 7 minutes. Then, the glass slide was turned by 140 degrees around the cell arrangement portion thereof, using the cell arrangement portion of the support as center of revolution and the glass slide was subjected to pulsed field gel electrophoresis for 7 minutes in the same manner. After the electrophoresis, SYBR Gold, which is a DNA fluorescent staining reagent, was dripped onto the cell-smeared position on the glass slide to stain the DNA. The stained DNA was observed through a fluorescence microscope.

**[0120]**

## (2) Results

FIG. 7 shows the results of microscopic observation of the DNA

subjected to two-dimensional pulsed field gel electrophoresis by means of the above described method. In FIG. 7, the arrows indicate long chain DNA fragments that cannot be observed by one-dimensional pulsed field gel electrophoresis. Because such long chain DNA fragments can be  
5 observed by the method of the present invention, separation of nucleic acid by two-dimensional pulsed field gel electrophoresis according to the present invention allows detection of cells in which DNA in the cell nucleus is fragmented with a high degree of sensitivity if compared with separation of nucleic acid by one-dimensional pulsed field gel  
10 electrophoresis. In particular, since DNA fragmentation takes place randomly, if the DNA in the cell nucleus is fragmented or not cannot be verified unless long chain DNA fragments are observable. As shown in the lower half of FIG. 8 that schematically illustrates DNA fragmentation, it is conceivable that long chain DNA fragmentation occurs first (d in FIG.  
15 8) and subsequently short chain DNA fragmentation occurs (a, b in FIG. 8) thanks to the long chain DNA fragmentation so that eventually DNA particles (c in FIG. 8) are produced in a mode of DNA fragmentation. In this mode, if short chain DNA fragments (a, b) and DNA particles (c) are undetectable by one-dimensional pulsed field gel electrophoresis, cells that  
20 are in the initial stages of DNA fragmentation cannot be observed provided that long chain DNA fragments (d) are undetectable. Separation of nucleic acid by two-dimensional pulsed field gel electrophoresis according to the present invention dissolves this problem of one-dimensional pulsed field gel electrophoresis. Thus, as two-dimensional pulsed field gel  
25 electrophoresis according to the present invention has made it possible to observe long chain DNA fragments (d) that are not observable by conventional one-dimensional pulsed field gel electrophoresis, cells in the initial stages of DNA fragmentation can be observed with a high degree of sensitivity.

## CLAIMS

1. A method of detecting a nucleic acid cleavage contained in a single cell, comprising the following steps (1) through (5):

5 (1) a step of arranging one or more cells on a support having an amino group on the surface thereof;

(2) a step of covering the cell or cells with a mixture of purified proteolytic enzyme having a weakly alkaline optimum pH value and a weakly acidic solution containing a gelling agent;

(3) a step of solidifying the weakly acidic solution;

10 (4) a step of immersing the support carrying thereon the solidified weakly acidic solution into a weakly alkaline solution containing a condensed phosphoric acid compound, a surface active agent and a reducing agent; and

(5) a step of separating the nucleic acid derived from the cell or 15 cells on the support immersed in the weakly alkaline solution by means of pulse-field gel electrophoresis using a solution for electrophoresis containing a condensed phosphoric acid compound.

2. A method of manufacturing a cell that is free from nucleic acid cleavage, comprising the following steps (1) through (6):

20 (1) a step of arranging one or more cells found in a test sample on a support having an amino group on the surface thereof;

(2) a step of covering the cell or cells with a mixture of purified proteolytic enzyme having a weakly alkaline optimum pH value and a weakly acidic solution containing a gelling agent;

25 (3) a step of solidifying the weakly acidic solution;

(4) a step of immersing the support carrying thereon the solidified weakly acidic solution into a weakly alkaline solution containing a condensed phosphoric acid compound, a surface active agent and a

reducing agent;

(5) a step of separating the nucleic acid derived from the cell or cells on the support immersed in the weakly alkaline solution by means of pulse-field gel electrophoresis using a solution for electrophoresis

5 containing a condensed phosphoric acid compound; and

(6) a step of determining the cell or cells in the test sample as cell or cells that are free from nucleic acid cleavage when the mode of separation of the nucleic acid that has been subjected to electrophoresis is substantially similar to the mode of separation of the nucleic acid derived  
10 from a normal cell or cells.

3. The method according to claim 1 or 2, wherein the purified proteolytic enzyme having a weakly alkaline optimum pH value is active for dissociation of the peptide bond formed between arginine and arbitrary amino acid and/or the peptide bond between lysine and arbitrary amino  
15 acid.

4. The method according to claim 1 or 2, wherein the purified proteolytic enzyme having a weakly alkaline optimum pH value is a purified trypsin-like enzyme.

5. The method according to claim 1 or 2, wherein the condensed  
20 phosphoric acid compound is at least a phosphoric acid compound selected from a group consisting of polyphosphoric acid, hexametaphosphoric acid and salts thereof.

6. The method according to claim 1 or 2, wherein the pulsed field gel electrophoresis is achieved by an electrophoresis apparatus having two  
25 or more pairs of electrodes, the two or more pairs of electrodes, being arranged so as to intersect each other at an angle between 45 degrees and 120 degrees.

7. The method according to claim 1 or 2, wherein the pulsed field gel electrophoresis is achieved by executing the first pulsed field gel

electrophoresis, turning the support by 60 to 160 degrees around the cell arrangement portion thereof, using the cell arrangement portion as center of revolution, and executing the second pulsed field gel electrophoresis.

8. The method according to claim 1 or 2, wherein the cell or cells  
5 are sperm cell or sperm cells.

9. A kit for detecting nucleic acid cleavage or for manufacturing a cell with cleavage-free nucleic acid, comprising the following components (1) through (5):

(1) a support having an amino group on the surface thereof;

10 (2) purified proteolytic enzyme having a weakly alkaline optimum pH value;

(3) a weakly acidic solution containing a gelling agent;

(4) a weakly alkaline solution containing a condensed phosphoric acid compound, a surface active agent and a reducing agent;  
15 and

(5) a solution for electrophoresis containing a condensed phosphoric acid compound.

10. The kit according to claim 9, wherein the kit further comprises the following component (6):

20 (6) an electrophoresis apparatus having two or more pairs of electrodes, the two or more pairs of electrodes being arranged so as to intersect each other at an angle between 45 degrees and 90 degrees.