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(54) 【発明の名称】 早発的染色体凝縮を誘導するための材料及び方法

(57) 【要約】

本発明は、未刺激細胞を使って、染色体異常を研究するための簡易且つ迅速な方法を提供する。早発性染色体凝縮 (PCC) は、有糸分裂増強因子の存在下で未刺激細胞をインキュベートすることによって誘導される。本発明の方法は、細胞の刺激又は有糸分裂細胞との融合を伴い、従来技術の方法に比べて迅速である。本発明の方法によって生産された凝縮された染色体は、多種類の細胞遺伝学的分析、特にインシツハイブリダイゼーションプローブ及び染色体ペインティングと共に使用され得る。本技法は、均一な全身低LET (線形エネルギートランスファー) 暴露 (uniform whole-body low-linear energy transfer exposure) を含む放射線被曝の生物学的線量測定に応用することができる。

【特許請求の範囲】

【請求項 1】

有糸分裂増強特性を有するサイクリンキナーゼを含む、細胞における早発的染色体凝縮を誘導するための培地であって、サイクリンキナーゼが、早発的染色体凝縮を誘導する有効量で存在する、前記培地。

【請求項 2】

サイクリンキナーゼが、 $p34^{cdc2}$ / サイクリン B キナーゼである、請求項 1 に記載の培地。

【請求項 3】

ホスファターゼ阻害剤を更に含む、請求項 1 に記載の培地。

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【請求項 4】

ホスファターゼ阻害剤が、オカダ酸、オカダ酸の塩、カリクリン、カンタリジン酸、カンタリジン、シペルメトリン、デルタメトリン、デホスタチン、3、4-デホスタチン、エンドタール、フェンバレート、ホストリエシン、ミクロシスチン-LA、ミクロシスチン-LF、ミクロシスチン-LR、ミクロシスチン-LW、ミクロシスチン-RR 及びミクロシスチン-YR から成る群より選択される、請求項 3 に記載の培地。

【請求項 5】

エネルギー源を更に含む、請求項 1 に記載の培地。

【請求項 6】

エネルギー源が、ATP 及び GTP から成る群より選択される、請求項 5 に記載の培地。

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【請求項 7】

トランスフェクション試薬を更に含む、請求項 1 に記載の培地。

【請求項 8】

請求項 1 乃至 7 のいずれか一項に記載の培地を含むキット。

【請求項 9】

染色体を分析する方法であって、

(a) 有糸分裂増強特性を有するサイクリンキナーゼを含む培地で細胞をインキュベートするステップであって、サイクリンキナーゼが、早発的染色体凝縮を誘導する有効量で存在する、前記ステップと、

(b) 凝縮した染色体を分析するステップと、

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を含む、前記方法。

【請求項 10】

化合物の染色体異常誘発作用を評価する方法であって、

(a) 細胞を化合物に接触させるステップと、

(b) 有糸分裂増強特性を有するサイクリンキナーゼを含む培地で細胞をインキュベートするステップであって、サイクリンキナーゼが、早発的染色体凝縮を誘導する有効量で存在する、前記ステップと、

(c) 切断、構造上及び / 又は数値的異常について凝縮した染色体を分析するステップと、

を含む、前記方法。

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【請求項 11】

細胞が、培地及び化合物に同時に接触せしめられる、請求項 10 に記載の方法。

【請求項 12】

化合物との接触後に、染色体修復を許容するための十分な時間にわたって、細胞をインキュベートするステップを更に含む、請求項 10 に記載の方法。

【請求項 13】

化合物の毒性を評価する方法であって、

(a) 細胞を化合物に接触させるステップと、

(b) 有糸分裂増強特性を有するサイクリンキナーゼを含む培地で細胞をインキュベートするステップであって、サイクリンキナーゼが、早発的染色体凝縮を誘導する有効量で存

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在する、前記ステップと、

(c)凝縮した染色体を分析するステップと、
を含む、前記方法。

【請求項14】

細胞が、培地及び化合物に同時に接触せしめられる、請求項13に記載の方法。

【請求項15】

化合物との接触後に、染色体修復を許容するための十分な時間にわたって、細胞をインキュベートするステップを更に含む、請求項13に記載の方法。

【請求項16】

被検体における染色体異常を検出する方法であって、

(a)被検体から一つ以上の細胞を単離するステップと、

(b)少なくとも一つ以上の細胞を、有糸分裂増強特性を有するサイクリンキナーゼを含む培地に接触させるステップであって、サイクリンキナーゼが、早発的染色体凝縮を誘導する有効量で存在する、前記ステップと、

(c)染色体異常について凝縮した染色体を分析するステップと、
を含む、前記方法。

【請求項17】

染色体異常が、細胞内で凝縮した染色体ドメイン又はスポットの数の評価に基づいて分析される、請求項16に記載の方法。

【請求項18】

被検体がインテロである、請求項17に記載の方法。

【請求項19】

異常が数値的な異常である、請求項17に記載の方法。

【請求項20】

異常が構造上の異常である、請求項17に記載の方法。

【請求項21】

被検体が受けた放射線量を評価する方法であって、

(a)被検体から一つ以上の細胞を単離するステップと、

(b)少なくとも一つ以上の細胞を、有糸分裂増強特性を有するサイクリンキナーゼを含む培地に接触させるステップであって、サイクリンキナーゼが、早発的染色体凝縮を誘導する有効量で存在する、前記ステップと、

(c)染色体異常について凝縮した染色体を分析するステップと、
を含む、前記方法。

【請求項22】

サイクリンキナーゼが、p34^{cdc2}/サイクリンBキナーゼである、請求項9、10、13、16又は21のいずれか一項に記載の方法。

【請求項23】

ホスファターゼ阻害剤を更に含む、請求項9、10、13、16又は21のいずれか一項に記載の方法。

【請求項24】

ホスファターゼ阻害剤が、オカダ酸、オカダ酸の塩、カリクリン、カンタリジン酸、カンタリジン、シペルメトリン、デルタメトリン、デホスタチン、3、4-デホスタチン、エンドタール、フェンバレート、ホストリエシン、ミクロシスチン-LA、ミクロシスチン-LF、ミクロシスチン-LR、ミクロシスチン-LW、ミクロシスチン-RR及びミクロシスチン-YRから成る群より選択される、請求項23に記載の方法。

【請求項25】

エネルギー源を更に含む、請求項9、10、13、16又は21のいずれか一項に記載の方法。

【請求項26】

エネルギー源が、ATP及びGTPから成る群より選択される、請求項25に記載の方法

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【請求項 27】

トランスフェクション試薬を更に含む、請求項 9、10、13、16 又は 21 のいずれか一項に記載の方法。

【請求項 28】

細胞がリンパ球である、請求項 9、10、13、16 又は 21 のいずれか一項に記載の方法。

【請求項 29】

細胞が哺乳動物細胞である、請求項 9、10、13、16 又は 21 のいずれか一項に記載の方法。

【請求項 30】

細胞がヒト末梢系血液リンパ球である、請求項 9、10、13、16 又は 21 のいずれか一項に記載の方法。

【請求項 31】

細胞が鼠細胞である、請求項 9、10、13、16 又は 21 のいずれか一項に記載の方法。

【請求項 32】

細胞が鼠末梢系血液リンパ球である、請求項 9、10、13、16 又は 21 のいずれか一項に記載の方法。

【請求項 33】

染色体を分析するステップが、染色体スプレッドを調製することを含む、請求項 9、10、13、16 又は 21 のいずれか一項に記載の方法。

【請求項 34】

染色体を分析するステップが、オリゴヌクレオチドを少なくとも一つの染色体にハイブリダイズさせ、且つ、染色体スポットを数えることを含む、請求項 9、10、13、16 又は 21 のいずれか一項に記載の方法。

【請求項 35】

オリゴヌクレオチドが、検出可能な成分を含む、請求項 34 に記載の方法。

【請求項 36】

検出可能な成分が蛍光成分である、請求項 35 に記載の方法。

【請求項 37】

検出可能な成分が、ビオチン、ジゴキシゲニン、抗原、酵素及びハプテンから成る群より選択される、請求項 35 に記載の方法。

【請求項 38】

有糸分裂増強特性を有するサイクリンキナーゼを含む細胞培地及び細胞を含む組成物であって、サイクリンキナーゼが、早発的染色体凝縮を誘導する有効量で存在する、前記組成物。

【請求項 39】

サイクリンキナーゼが、p34^{cdc2}/サイクリン B キナーゼである、請求項 38 に記載の組成物。

【請求項 40】

ホスファターゼ阻害剤を更に含む、請求項 38 に記載の組成物。

【請求項 41】

ホスファターゼ阻害剤が、オカダ酸、オカダ酸の塩、カリクリン、カンタリジン酸、カンタリジン、シベルメトリン、デルタメトリン、デホスタチン、3、4-デホスタチン、エンドタール、フェンバレート、ホストリエシン、ミクロシスチン-LA、ミクロシスチン-LF、ミクロシスチン-LR、ミクロシスチン-LW、ミクロシスチン-RR 及びミクロシスチン-YR から成る群より選択される、請求項 40 に記載の組成物。

【請求項 42】

エネルギー源を更に含む、請求項 38 に記載の組成物。

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【請求項 4 3】

エネルギー源が、ATP及びGTPから成る群より選択される、請求項 4 2 に記載の組成物。

【請求項 4 4】

トランスフェクション試薬を更に含む、請求項 3 8 に記載の組成物。

【請求項 4 5】

請求項 3 8 乃至 4 4 のいずれか一項に記載の培地を含むキット。

【発明の詳細な説明】

【発明者】

【0001】

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【発明の分野】

【0002】

本発明は、細胞遺伝学、分子細胞遺伝学、細胞生物学、遺伝毒性学と遺伝学の分野に関わる。特に、本発明は、早発的染色体凝縮を誘導する方法及び凝縮した染色体を使って遺伝物質を分析する方法に関わる。

【背景】

【0003】

種々の環境傷害は、遺伝物質に物理的損傷を誘導する可能性を持つ。環境毒素への被曝に加えて、人間の放射線への事故時被曝は、重大な関心事である。簡易で迅速な方法の開発は傷害線量評価のために必要である。そして、それは被曝した個人の処置のためになる。

【0004】

Muller 及び Streffer (Muller et al. (1991) Int. J. Radiat. Biol. 59 (863-873)) は、放射線障害の生物学的指標の広範囲にわたる再検討を発表した。そして、線量評価のための生物学的線量測定について現在の技術を説明した。高い放射線量に曝されると、日常的な分裂中期スプレッド染色体異常分析による線量評価のためには、利用可能な有糸分裂細胞の数が十分ではない。暴露された個人の血液リンパ球で実行される早発的染色体凝縮 (PCC) アッセイは、臨床学的に有意義である迅速な生物線量測定法として考察されている (Pantelias et al. (1985) Mutat. Res. 149, 67-72; Blakely et al. (1995) Stem Cells 13, 223-230; and Prasanna et al. (1997) Health Phys. 72, 594-600)。

【0005】

現在、染色体への物理的損傷は、分裂中期スプレッドを調製した後、染色体の観察によって分析できる。染色体は、短期の細胞培養の後の有糸分裂細胞で視覚化される。この細胞培養では、細胞は有糸分裂促進剤によって刺激されて増殖し、その後、コルヒチン又はコルセミドで細胞周期停止を受ける。染色体は、染色法又は蛍光プローブでハイブリッドすることによって処理された後に顕微鏡下で観察される。この技術は、細胞が増殖するように連続的に刺激することに依存し、有用な収率を得るには 48 時間以上にわたる細胞培養を必要とする。技術は、集中的労力を要し、実行するにあたって細胞遺伝学的技術経験を必要とする。この分析は、細胞の殺害及び処理によって誘導された細胞周期遅延によって、さらに複雑になる。それに加えて、凝縮された染色体の低い収率のため、統計学的に有意のデータを得るには、大量の分裂中期スプレッドが必要となる。

【0006】

染色体の物理的損傷を分析するもう一つの方法は、細胞で早発的染色体凝縮 (PCC) を誘導させて染色体スプレッドを調製することを含む。歴史的に、早発的染色体凝縮は、対象細胞を有糸分裂細胞と融合させることによって達成されてきた。これにより、試験細胞 (test cells) において染色体凝縮が染色質様構造になる結果となった。この技術により早発的染色体凝縮は起こるが、その実施と関連して幾らかの困難がある。この技術は、試験細胞と融合する有糸分裂細胞の安定した供給を必要とする。有糸分裂細胞の培養及び維持は、この方法に更なる費用を追加する。その上、細胞融合技術 (例えば、PEG 媒介融合

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)は、能率が悪くて、融合細胞の収率は低く変動的である。この結果、試験細胞での早発的染色体凝縮の収率は低くて変動することになる(Pantelias et al. (1983) Somatic Cell Genet. 9, 533-547)。

【0007】

早発的染色体凝縮を誘導する有糸分裂細胞融合の欠陥は、本技術分野で周知であり、代替する簡易且つ迅速なプロトコルの探索は現在の研究のトピックである(Gotoh et al. (1996) Int. J. Radiat. Biol. 70, 517-520; Kanda et al. (1999) Int. J. Radiat. Biol. 75, 441-446; Durante et al. (1998) Int. J. Radiat. Biol. 74, 457-462; and Coco-Martin et al. (1997) Int. J. Radiat. Biol. 71, 265-273)。最近、早発的染色体凝縮は、有糸分裂促進剤で細胞を刺激した後、ホスファターゼ阻害剤の存在下で細胞を培養することによって誘導された。1型及び2A型タンパク質ホスファターゼの阻害剤は、増殖細胞でPCCを誘導するのに用いられた(Gotoh et al. (1996) Int. J. Radiat. Biol. 70, 517-520; Kanda et al. (1999) Int. J. Radiat. Biol. 75, 441-446; Durante et al. (1998) Int. J. Radiat. Biol. 74, 457-462; and Coco-Martin et al. (1997) Int. J. Radiat. Biol. 71, 265-273)。

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【0008】

ホスファターゼ阻害剤処理によって調製される凝縮された染色体は、PCCスプレッドでの染色体異常分析を使うことによって、生物学的な線量測定応用のために評価された。早発的染色体凝縮は、有糸分裂促進剤で刺激された細胞において、オカダ酸(OA)(Gotoh et al. (1996) Int. J. Radiat. Biol. 70, 517-520; Kanda et al. (1999) Int. J. Radiat. Biol. 75, 441-446)又はカリクリンA(Durante et al. (1998) Int. J. Radiat. Biol. 74, 457-462)によって誘導され、有糸分裂促進剤刺激から48時間で得られた。ドウランテラ(Durante et al. (1998) Int. J. Radiat. Biol. 74, 457-462)は、200-kVp X線に曝された後の全染色体プローブ蛍光インシツハイブリダイゼーション(FISH)を用いて、G1及びM期の染色体異常の同時測定が可能であることを証明した。また、OA又はカリクリンAを含んでいる細胞培地で活発に分割している腫瘍株をインキュベーションすると、PCC誘導が起こることは示されている(Coco-Martin et al. (1997) Int. J. Radiat. Biol. 71, 265-273)。全染色体特異的プローブを使うことにより、放射線誘導染色体異常を含む化学的に誘導されたPCCスプレッドを、2つよりも多い染色体スポットを持つ細胞として、容易に同定することができる。放射線感受性の相違は、放射線感受性細胞株(SCC61)と放射線抵抗性細胞株(A549)との間で示された(Coco-Martin et al. (1997) Int. J. Radiat. Biol. 71, 265-273)。

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【0009】

ホスファターゼ阻害剤の使用により、刺激された細胞又は増殖している細胞で早発的染色体凝縮は起こるが、現在利用可能な方法は、染色体異常分析に有用な早発的染色体凝縮が十分に高い収率で生じるような培養期間を要求する。

【発明の簡単な概要】

【0010】

先に議論された方法にもかかわらず、本技術分野では、環境傷害による遺伝物質の損傷を評価するための迅速且つ簡易な方法に対する要求がある。現在、このような評価をすることの困難性の主な原因は、凝縮された染色体をその後の分析のために生成するのに必要とされる時間及び労力である。本発明は、未刺激細胞において、早発的染色体凝縮を迅速に且つ高収率で誘導する細胞培地を提供することによって、長く渴望されてきた要求に応ずる。本発明は、未刺激細胞において早発的染色体凝縮を誘導する細胞融合の必要性に対処し、且つ、本技術分野で知られる他の方法によって要求される刺激及びその後のインキュベーションの必要性に対処する。本発明の材料及び方法を使うことによって調製された凝縮された染色体は、化学的に誘導されたPCCスプレッドで全染色体特異的プローブを用いたFISHによって未刺激のHPBLでの特異的染色体への損傷を研究することができるということを示すために使われてきた。本発明の方法は、本技術分野で知られるものよりも簡易且つ迅速であり、オートメーション化した高スループットの染色体損傷アッセイ

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に適している。これらの方法には、迅速な生物学的線量測定を含む多数の応用がある。

【0011】

本発明は、細胞における早発的染色体凝縮を誘導するための培地を提供する。好ましい実施形態では、細胞培地は、一つ以上の有糸分裂増強因子を含む。幾つかの実施形態では、有糸分裂増強因子は、一つ以上のサイクリン、サイクリンキナーゼ、ヒストンキナーゼ、サイクリン、トポイソメラーゼ、染色体構造維持(SMC)タンパク質、ヒストン、cdk1基質及び有糸分裂増強因子の成分である。好ましい実施形態では、有糸分裂増強因子は、p34^{cdc2}/サイクリンBキナーゼである。

【0012】

本発明の細胞培地は、ホスファターゼ阻害剤を含んでいてよい。この場合、ホスファターゼ阻害剤は、オカダ酸、オカダ酸の塩、カリクリン、カンタリジン酸、カンタリジン、シベルメトリン、デルタメトリン、デホスタチン、3、4-デホスタチン、エンドタール、フェンバレート、ホストリエシン、ミクロシスチン-LA、ミクロシスチン-LF、ミクロシスチン-LR、ミクロシスチン-LW、ミクロシスチン-RR及びミクロシスチン-YRの一つ以上を含むことができる。本発明の細胞培地は、エネルギー源(好ましくは、ATP及び/又はGTP)を含んでよい。

【0013】

本発明は、有糸分裂増強因子を含む培地であって早発的染色体凝縮を誘導するものを用いて細胞をインキュベートするステップと、凝縮した染色体を分析するステップと、を含む、染色体を分析する方法を提供する。幾つかの実施形態では、有糸分裂増強因子は、サイクリンキナーゼ、ヒストンキナーゼ、サイクリン、トポイソメラーゼ、SMCタンパク質、cdk1基質、ヒストン及び有糸分裂増強因子(MPF)の成分である。幾つかの好ましい実施形態では、有糸分裂増強因子は、p34^{cdc2}/サイクリンBキナーゼを含む。

【0014】

染色体を分析する方法に使用される培地は、ホスファターゼ阻害剤を含んでいてよい。好ましくは、ホスファターゼ阻害剤は、オカダ酸、オカダ酸の塩、カリクリン、カンタリジン酸、カンタリジン、シベルメトリン、デルタメトリン、デホスタチン、3、4-デホスタチン、エンドタール、フェンバレート、ホストリエシン、ミクロシスチン-LA、ミクロシスチン-LF、ミクロシスチン-LR、ミクロシスチン-LW、ミクロシスチン-RR及びミクロシスチン-YRの一つ以上を含むことができる。培地は、エネルギー源(好ましくは、ATP及び/又はGTP)を含んでよい。培地は、トランスフェクション試薬を含んでよい。

【0015】

染色体の分析方法は、いかなる種類の細胞でも実施されてよい。幾つかの実施形態では、細胞はリンパ球である。好ましくは、細胞は哺乳動物細胞である。幾つかの実施形態では、細胞はヒト末梢系血液リンパ球である。幾つかの実施形態では、細胞は鼠細胞であり、好ましくは、鼠末梢系血液リンパ球である。

【0016】

染色体の分析方法は、染色体スプレッドを調製することを含んでよい。該方法は、一つ以上のオリゴヌクレオチドを一つ以上の染色体にハイブリダイズさせ、且つ、染色体スポットを数えることを含むことができる。幾つかの実施形態では、一つ以上のオリゴヌクレオチドは検出可能な成分を含んでよい。好ましくは、検出可能な成分は蛍光成分であるが、ビオチン、ジゴキシゲニン、抗原、酵素及びハプテンの一つ以上であってもよい。

【0017】

また、本発明は、化合物の染色体異常誘発作用を評価する方法であって、細胞を化合物に接触させるステップと、有糸分裂増強因子を含む培地であって早発的染色体凝縮を誘導するもので細胞をインキュベートするステップと、切断、構造上及び/又は数値的な異常について凝縮した染色体を分析するステップとを含む方法を提供する。幾つかの実施形態では、細胞は、培地及び化合物に同時に接触せしめられる。その他の実施形態では、細胞は

、化合物に接触せしめられた後に適切な培地に移されてもよい。幾つかの場合においては、化合物との接触後に、染色体修復を許容するための十分な時間にわたって、細胞をインキュベートすることが望ましい。幾つかの実施形態では、有糸分裂増強因子は、サイクリンキナーゼ、ヒストンキナーゼ、サイクリン、トポイソメラーゼ、SMCタンパク質、cdk1基質、ヒストン及び有糸分裂増強因子(MPF)の成分である。幾つかの好ましい実施形態では、有糸分裂増強因子は、p34^{cdc2}/サイクリンBキナーゼを含む。

【0018】

化合物の染色体異常誘発作用を評価する方法に使用される培地は、ホスファターゼ阻害剤を含んでいてよい。好ましくは、ホスファターゼ阻害剤は、オカダ酸、オカダ酸の塩、カリクリンA、カンタリジン酸、カンタリジン、シベルメトリン、デルタメトリン、デホスタチン、3、4-デホスタチン、エンドタール、フェンバレート、ホストリエシン、ミクロシスチン-LA、ミクロシスチン-LF、ミクロシスチン-LR、ミクロシスチン-LW、ミクロシスチン-RR及びミクロシスチン-YRの一つ以上を含むことができる。培地は、エネルギー源(好ましくは、ATP及び/又はGTP)を含んでよい。培地は、トランスフェクション試薬を含んでよい。

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【0019】

化合物の染色体異常誘発作用を評価する方法は、いかなる種類の細胞でも実施されてよい。幾つかの実施形態では、細胞はリンパ球である。好ましくは、細胞は哺乳動物細胞である。幾つかの実施形態では、細胞はヒト末梢系血液リンパ球である。幾つかの実施形態では、細胞は鼠細胞であり、好ましくは、鼠末梢系血液リンパ球である。

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【0020】

化合物の染色体異常誘発作用を評価する方法は、染色体スプレッドを調製することを含んでよい。該方法は、一つ以上のオリゴヌクレオチドを一つ以上の染色体にハイブリダイズさせ、且つ、染色体スポットを数えることを含むことができる。幾つかの実施形態では、一つ以上のオリゴヌクレオチドは検出可能な成分を含んでよい。好ましくは、検出可能な成分は蛍光成分であるが、ビオチン、ジゴキシゲニン、抗原、酵素及びハプテンの一つ以上であってもよい。

【0021】

また、本発明は、化合物の毒性を評価する方法であって、細胞を化合物に接触させるステップと、有糸分裂増強因子を含む培地であって早発的染色体凝縮を誘導するもので細胞をインキュベートするステップと、凝縮した染色体を分析するステップとを含む方法を提供する。幾つかの実施形態では、細胞は、培地及び化合物に同時に接触せしめられる。その他の実施形態では、細胞は、化合物に接触せしめられた後に適切な培地に移されてもよい。幾つかの場合においては、化合物との接触後に、染色体修復を許容するための十分な時間にわたって、細胞をインキュベートすることが望ましい。幾つかの実施形態では、有糸分裂増強因子は、サイクリンキナーゼ、ヒストンキナーゼ、サイクリン、トポイソメラーゼ、SMCタンパク質、cdk1基質、ヒストン及び有糸分裂増強因子(MPF)の成分である。幾つかの好ましい実施形態では、有糸分裂増強因子は、p34^{cdc2}/サイクリンBキナーゼを含む。

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【0022】

化合物の毒性を評価する方法に使用される培地は、ホスファターゼ阻害剤を含んでいてよい。好ましくは、ホスファターゼ阻害剤は、オカダ酸、オカダ酸の塩、カリクリンA、カンタリジン酸、カンタリジン、シベルメトリン、デルタメトリン、デホスタチン、3、4-デホスタチン、エンドタール、フェンバレート、ホストリエシン、ミクロシスチン-LA、ミクロシスチン-LF、ミクロシスチン-LR、ミクロシスチン-LW、ミクロシスチン-RR及びミクロシスチン-YRの一つ以上を含むことができる。培地は、エネルギー源(好ましくは、ATP及び/又はGTP)を含んでよい。培地は、トランスフェクション試薬を含んでよい。

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【0023】

化合物の毒性を評価する方法は、いかなる種類の細胞でも実施されてよい。幾つかの実施

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形態では、細胞はリンパ球である。好ましくは、細胞は哺乳動物細胞である。幾つかの実施形態では、細胞はヒト末梢系血液リンパ球である。幾つかの実施形態では、細胞は鼠細胞であり、好ましくは、鼠末梢系血液リンパ球である。

【0024】

化合物の毒性を評価する方法は、染色体スプレッドを調製することを含んでよい。該方法は、一つ以上のオリゴヌクレオチドを一つ以上の染色体にハイブリダイズさせ、且つ、染色体スポットを数えることを含むことができる。幾つかの実施形態では、一つ以上のオリゴヌクレオチドは検出可能な成分を含んでよい。好ましくは、検出可能な成分は蛍光成分であるが、ビオチン、ジゴキシゲニン、抗原、酵素及びハプテンの一つ以上であってもよい。

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【0025】

また、本発明は、被検体における染色体異常を検出する方法であって、被検体から一つ以上の細胞を単離するステップと、少なくとも一つ以上の細胞を、有糸分裂増強因子を含む培地であって早発的染色体凝縮を誘導するものに接触させるステップと、染色体異常について凝縮した染色体を分析するステップとを含む方法を提供する。幾つかの実施形態では、有糸分裂増強因子は、サイクリンキナーゼ、ヒストンキナーゼ、サイクリン、トポイソメラーゼ、SMCタンパク質、cdk1基質、ヒストン及び有糸分裂増強因子(MPF)の成分である。幾つかの好ましい実施形態では、有糸分裂増強因子は、p34^{cdc2}/サイクリンBキナーゼを含む。

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【0026】

被検体における染色体異常を検出する方法に使用される培地は、ホスファターゼ阻害剤を含んでいてよい。好ましくは、ホスファターゼ阻害剤は、オカダ酸、オカダ酸の塩、カリクリンA、カンタリジン酸、カンタリジン、シペルメトリン、デルタメトリン、デホスタチン、3、4-デホスタチン、エンドタール、フェンバレート、ホストリエシン、ミクロシスチン-LA、ミクロシスチン-LF、ミクロシスチン-LR、ミクロシスチン-LW、ミクロシスチン-RR及びミクロシスチン-YRの一つ以上を含むことができる。培地は、エネルギー源(好ましくは、ATP及び/又はGTP)を含んでよい。培地は、トランスフェクション試薬を含んでよい。

【0027】

被検体における染色体異常を検出する方法は、いかなる種類の細胞でも実施されてよい。幾つかの実施形態では、細胞はリンパ球である。好ましくは、細胞は哺乳動物細胞である。幾つかの実施形態では、細胞はヒト末梢系血液リンパ球である。幾つかの実施形態では、細胞は鼠細胞であり、好ましくは、鼠末梢系血液リンパ球である。幾つかの実施形態では、細胞は、被検体からインキュテロで得られる。

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【0028】

被検体における染色体異常を検出する方法は、染色体スプレッドを調製することを含んでよい。該方法は、一つ以上のオリゴヌクレオチドを一つ以上の染色体にハイブリダイズさせ、且つ、染色体スポットを数えることを含むことができる。幾つかの実施形態では、一つ以上のオリゴヌクレオチドは検出可能な成分を含んでよい。好ましくは、検出可能な成分は蛍光成分であるが、ビオチン、ジゴキシゲニン、抗原、酵素及びハプテンの一つ以上

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【0029】

また、本発明は、被検体が受けた放射線量を評価する方法であって、被検体から一つ以上の細胞を単離するステップと、少なくとも一つ以上の細胞を、有糸分裂増強因子を含む培地であって早発的染色体凝縮を誘導するものに接触させるステップと、切断、構造上及び/又は数値的な異常について凝縮した染色体を分析するステップとを含む方法を提供する。幾つかの実施形態では、有糸分裂増強因子は、サイクリンキナーゼ、ヒストンキナーゼ、サイクリン、トポイソメラーゼ、SMCタンパク質、cdk1基質、ヒストン及び有糸分裂増強因子(MPF)の成分である。幾つかの好ましい実施形態では、有糸分裂増強因子は、p34^{cdc2}/サイクリンBキナーゼを含む。

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【0030】

被検体が受けた放射線量を評価する方法に使用される培地は、ホスファターゼ阻害剤を含んでよい。好ましくは、ホスファターゼ阻害剤は、オカダ酸、オカダ酸の塩、カリクリンA、カンタリジン酸、カンタリジン、シペルメトリン、デルタメトリン、デホスタチン、3、4-デホスタチン、エンドタール、フェンバレート、ホストリエシン、ミクロシスチン-LA、ミクロシスチン-LF、ミクロシスチン-LR、ミクロシスチン-LW、ミクロシスチン-RR及びミクロシスチン-YRの一つ以上を含むことができる。培地は、エネルギー源（好ましくは、ATP及び/又はGTP）を含んでよい。培地は、トランスフェクション試薬を含んでよい。

【0031】

被検体が受けた放射線量を評価する方法は、いかなる種類の細胞でも実施されてよい。幾つかの実施形態では、細胞はリンパ球である。好ましくは、細胞は哺乳動物細胞である。幾つかの実施形態では、細胞はヒト末梢系血液リンパ球である。幾つかの実施形態では、細胞は鼠細胞であり、好ましくは、鼠末梢系血液リンパ球である。

【0032】

被検体が受けた放射線量を評価する方法は、染色体スプレッドを調製することを含んでよい。該方法は、一つ以上のオリゴヌクレオチドを一つ以上の染色体にハイブリダイズさせ、且つ、染色体スポットを数えることを含むことができる。幾つかの実施形態では、一つ以上のオリゴヌクレオチドは検出可能な成分を含んでよい。好ましくは、検出可能な成分は蛍光成分であるが、ビオチン、ジゴキシゲニン、抗原、酵素及びハプテンの一つ以上であつてもよい。

【0033】

また、本発明は、細胞及び細胞培地を含む組成物を提供する。ここで、細胞培地は、有糸分裂増強因子を含み、細胞において早発的染色体凝縮を誘導する。本発明の組成物では、有糸分裂増強因子は、サイクリンキナーゼ、ヒストンキナーゼ、サイクリン、トポイソメラーゼ、染色体構造維持(SMC)タンパク質、ヒストン、cdk1基質及び有糸分裂増強因子の成分の一つ以上であつてよい。幾つかの好ましい実施形態では、有糸分裂増強因子は、p34^{cdc2}/サイクリンBキナーゼを含む。本発明の組成物は、ホスファターゼ阻害剤を含んでよい。ホスファターゼ阻害剤は、オカダ酸、オカダ酸の塩、カリクリンA、カンタリジン酸、カンタリジン、シペルメトリン、デルタメトリン、デホスタチン、3、4-デホスタチン、エンドタール、フェンバレート、ホストリエシン、ミクロシスチン-LA、ミクロシスチン-LF、ミクロシスチン-LR、ミクロシスチン-LW、ミクロシスチン-RR及びミクロシスチン-YRの一つ以上を含むことができる。培地は、エネルギー源（好ましくは、ATP及び/又はGTP）を含んでよい。

【0034】

本発明は、試験細胞において早発的染色体凝縮を誘導するためのキットを提供する。幾つかの実施形態では、キットは、有糸分裂増強因子を含み、細胞において早発的染色体凝縮を誘導する細胞培地の容器を一つ以上備えていてよい。有糸分裂増強因子は、サイクリンキナーゼ、ヒストンキナーゼ、サイクリン、トポイソメラーゼ、染色体構造維持(SMC)タンパク質、ヒストン、cdk1基質及び有糸分裂増強因子の成分の一つ以上であつてよい。幾つかの好ましい実施形態では、有糸分裂増強因子は、p34^{cdc2}/サイクリンBキナーゼを含む。本発明のキットは、一つ以上のホスファターゼ阻害剤を保持する一つ以上の容器を備えてよい。ホスファターゼ阻害剤は、オカダ酸、オカダ酸の塩、カリクリンA、カンタリジン酸、カンタリジン、シペルメトリン、デルタメトリン、デホスタチン、3、4-デホスタチン、エンドタール、フェンバレート、ホストリエシン、ミクロシスチン-LA、ミクロシスチン-LF、ミクロシスチン-LR、ミクロシスチン-LW、ミクロシスチン-RR及びミクロシスチン-YRの一つ以上を含むことができる。キットは、また、エネルギー源（好ましくは、ATP及び/又はGTP）を保持する一つ以上の容器をも備えていてよい。本発明のキットは、一つ以上のトランスフェクション試薬を保持する一つ以上の容器を備えていてよい。

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【0035】

本発明は、有糸分裂促進因子で細胞を刺激する必要なしに、細胞で早発的染色体凝縮を誘導するための材料及び方法を提供する。さらに、本発明は、早発的染色体凝縮を誘導することによる遺伝物質を分析する方法と、凝縮した染色体の物理的構造を分析する方法を提供する。本発明は、試験細胞で早発的染色体凝縮を必要とするどんな適用にも有用である。本発明は、特に細胞遺伝学、分子細胞遺伝学、細胞生物学、遺伝毒性学及び遺伝学の分野で有用である。

【0036】

幾つかの態様において、本発明は、診断細胞遺伝学で有用な材料と方法を提供する。本発明の材料と方法は、試験細胞の遺伝物質を評価するために、出産前、生後、およびプレ体内移植検定で使用され得る。例えば、本明細書で記述される方法は、精液で染色体異常の有無を決定するために潜在的な精液ドナーで遺伝物質を評価するのに用いられ得る。同様に、本発明は、インキュテロで被検者の遺伝物質を分析するために用いられ得る。

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【0037】

関連した幾つかの態様においては、本発明は、細胞遺伝学的研究において使用できる。例えば、遺伝学の分野で、本発明は、染色体異常（例えば、ダウン症候群）によって特徴づけられる種々の症候群と関連した遺伝子を検出するために使用される。特定の実施形態では、本発明は、ミクロ欠損症候群と関連する遺伝子の検出に使用される。もう一つの実施形態では、本発明は、癌と関連する（数と構造上の）染色体異常の検出に使用される。幾つかの好ましい実施形態では、本発明は、遺伝子増幅の検出に使用される。

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【0038】

環境試験の分野では、本発明は、被検者の環境傷害への照射の評価に使用される。幾つかの好ましい実施形態では、被検者が受ける線量の評価に使用される。放射線量は、事故被曝の結果として受け取られるか、職業上被曝の結果として受け取られる。本発明は、特に多数の被検者の照射の場合に有用である。本発明が自動化できる能力は、高スループットの自動化スクリーニングシステムに適しているからである。他の実施形態では、染色体異常を誘導する化合物への被検者の照射が評価され得る。

【0039】

幾つかの好ましい実施形態では、本発明は、薬の毒性を評価する方法を提供する。これらの方法は、染色体切断を誘導することができる試薬を有することが望ましい場合において、可能性のある化学療法剤を確認するのに有用である。この態様では、本発明の方法は、特定の試剤のクラストゲニシティ（染色体破壊能力）の評価に使用される。また、本発明の方法は、治療薬が染色体異常を誘発するかどうか決定するための初期的安全スクリーンとして使われる。

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【0040】

細胞

本発明の実施には、遺伝物質を有するいかなる種類の細胞を使用してもよい。例えば、細胞源として心臓、肺、肝臓、腎臓その他の組織からの細胞を使用できる。種々の組織からの細胞の単離は、当業者に既知のいかなる手法を使用しても達成される。好ましい実施形態では、細胞は、ヒト又は鼠細胞等の哺乳類起源のものである。幾つかの好ましい実施形態では、早発的染色体凝縮及び分析のために末梢血液リンパ球を使用してもよい。他の好ましい実施形態では、卵母細胞、又は、胎児、羊水又は株化細胞系統（幹細胞等）から得られたものでよい。

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【0041】

本発明に使われる細胞の単離は、当業者に既知のいかなる手段であってもよい。幾つかの好ましい実施形態では、末梢血液リンパ球（HPBLs）が使用される。末梢血液リンパ球の単離は、本分野では日常的である。一つの適当なプロトコールが下記に記載されており、当業者に既知の他のプロトコールも使用できる。以下のプロトコールでは、ヒト被検体から末梢血液リンパ球が単離される。末梢血液リンパ球は、同等に、いかなる被検体からも単離される。幾つかの好ましい実施形態では、被検体は、哺乳動物である。他の好ま

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しい実施形態では、被検体は、ヒト又はマウスである。

【0042】

リンパ球は、当業者に既知の適当な手法を使用して、全血液サンプルから単離される。適当な手法の例としては、例えば、Histopaque 1077 (Sigma Chemical Co.) を使用する密度勾配遠心法が挙げられる。遠心の後、細胞を収集してリン酸緩衝生理食塩水 (pH 7.0) で二回洗浄する。その後、適当な細胞培地に細胞を再懸濁させる。所与の種類 of 細胞に対して適当な細胞培地を選択することは、当業者にとって日常的である。細胞がリンパ球である場合、適当な培地は Karyomax (Life Technologies Inc.) である。引き続き分析に適当な濃度で細胞を再懸濁させる。例えば、約 1×10^6 細胞/ml から約 1.5×10^6 細胞/ml の濃度である。

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【0043】

細胞培地

本発明は、試験細胞で早発的染色体凝縮を誘導させる細胞培地を提供する。いかなる適当な細胞培地も一つ以上の有糸分裂増強因子で補充されて、本発明の細胞培地として使用される。適当な細胞培地は、早発的染色体凝縮の誘導期間にわたって対象の細胞を成育可能な状態にて維持できるものである。任意に、適当な細胞培地は、遷延性期間にわたって試験細胞を維持できるものである。

【0044】

本発明の細胞培地は、一般的に、試験細胞の生育力を維持するために選択された種々の成分を含む。このような成分は、アミノ酸、ビタミン、無機塩、緩衝液又は緩衝液塩、糖、脂質、微量元素、サイトカイン及びホルモンを含むが、これらに限定されない。適当な細胞培地は、例えば Life Technologies Inc. から市販されている。

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【0045】

好ましい実施形態では、本発明の細胞培地は、一つ以上の有糸分裂増強因子を含む。有糸分裂増強因子は、有糸分裂への細胞周期の進行に関連した試薬である。有糸分裂増強因子は、サイクリン、サイクリンキナーゼ、ヒストンキナーゼ、トポイソメラーゼ、SMCタンパク質、cdk1基質、ヒストン、及び有糸分裂促進因子(MPF)の成分を含むが、これらに限定されない。好ましい実施形態では、有糸分裂増強因子は、精製された有糸分裂増強因子であってよい。有糸分裂増強因子は、所望の純度レベルに精製され得る。好ましくは、有糸分裂増強因子は、少なくとも50%の純度(つまり、有糸分裂増強因子が、細胞培地に加えられる有糸分裂増強因子含有物質の重量の少なくとも50%までを成す)である。他の好ましい実施形態では、有糸分裂増強因子は、75%以上の純度、80%以上の純度、85%以上の純度、90%以上の純度又は85%以上の純度である。好ましい実施形態では、本発明の細胞培地は、p34^{cdc2}/サイクリンBキナーゼを含んでよい。適当なp34^{cdc2}/サイクリンBキナーゼは、例えばNew England Biolabsから市販されている。

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【0046】

有糸分裂増強因子は、単独で又は他の因子との組み合わせで、培地に加えられる。有糸分裂増強因子は、天然タンパク質型であっても、突然変異タンパク質型であってもよい。例えば、有糸分裂増強因子を含む融合タンパク質を使うことができる。融合タンパク質を製造するために、有糸分裂増強因子は、タンパク質又は他のタンパク質のペプチド部分とフレームに配置され得る。融合タンパク質の構築は、本技術分野では日常的である(例えば、Sambrookら、(1989)Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Pressを参照)。好ましい実施形態では、本発明の融合タンパク質は、有糸分裂増強因子に加えて、融合タンパク質、核局在化シグナル、精製標識、エピトープ等の細胞アップテイクを容易にするために、レセプタに対する一つ以上のリガンドを含んでよい。好ましい実施形態では、本発明の細胞培地は、有糸分裂増強因子及び核局在化配列を含む融合タンパク質を含む。適当な核局在化シグナルは、本技術分野では既知であり、例えば、米国特許第60

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51429号及び5736392号に見出される。

【0047】

有糸分裂増強因子に加えて、本発明の細胞培地は、ATP及びGTPに限定されない、一つ以上のエネルギー源を含んでよい。

【0048】

本発明の細胞培地は、一つ以上のトランスフェクション試薬を任意に含んでよい。本明細書では、トランスフェクション試薬とは、細胞培地に加えられた場合に、試験細胞による有糸分裂増強因子のアップテイクを向上させるいかなる試薬も含むと考えられる。トランスフェクション試薬には、中性脂質、カチオン性脂質、中性脂質とカチオン性脂質の混合物、タンパク質、ペプチド、リポタンパク質、リポペプチド等が含まれるが、これらに限定されない。適当なトランスフェクション試薬は、例えばPromega Inc.及びLife Technologies Incから市販されている。幾つかの好ましい実施形態では、本発明のトランスフェクション試薬は、レセプタ媒介エンドサイトーシスを向上させるペプチドを含んでよい。このようなトランスフェクション試薬の例として、米国特許第6103529号に見出せる。トランスフェクション試薬は、培地に直接的に加えられても、有糸分裂増強因子を培地へ加える前に有糸分裂増強因子と組み合わせてもよい。

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【0049】

本発明の細胞培地は、一つ以上のホスファターゼ阻害剤を任意に含んでよい。幾つかの好ましい実施形態では、タンパク質ホスファターゼ阻害剤は、セリン/スレオニンタンパク質ホスファターゼを特異的に阻害できる。幾つかの好ましい実施形態では、ホスファターゼ阻害剤は、タンパク質ホスファターゼ1及び2Aを特異的に阻害することができる。適当なホスファターゼ阻害剤には、オカダ酸、オカダ酸の塩、カリクリンA、カンタリジン酸、カンタリジン、シベルメトリン、デルタメトリン、デホスタチン、3、4-デホスタチン、エンドタール、フェンバレート、ホストリエシン、ミクロシスチン-LA、ミクロシスチン-LF、ミクロシスチン-LR、ミクロシスチン-LW、ミクロシスチン-RR及びミクロシスチン-YRが含まれるが、これらに限定されない。

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【0050】

細胞培養組成物

本発明の細胞培地は、細胞又は細胞群及び本発明の細胞培地を含む細胞培養組成物を調製するために使用され得る。細胞は、早発的染色体凝縮が誘導されるものであればいかなるものであってもよい。被検体から単離された細胞が特に好ましい。単離細胞は、被検体の器官又は組織に由来するものであってよく、血液、心臓、肺、上皮組織及び/又は小腸組織が含まれるが、これらに限定されない。

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【0051】

キット

本発明は、細胞遺伝学研究での使用に適用されるキットを意図する。一般的に、本発明のキットは、本発明の細胞培地を保持する一つ以上の容器を含んでよい。細胞培地は、液体の形態でもよいし、乾燥粉末濃縮物の形態でもよい。本発明のキットは、一つ以上の有糸分裂増強因子を保持する一つ以上の容器を含んでよい。この因子は、溶液中にあってもよいし、乾燥粉末の形態でもよい。本発明のキットは、一つ以上のホスファターゼ阻害剤を保持する一つ以上の容器を含んでよい。任意に、本発明のキットは、一つ以上のトランスフェクション試薬及び/又は一つ以上のエネルギー源を、溶液中に又は乾燥形態で保持する一つ以上の容器を含んでよい。

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【0052】

本発明のキットは、本発明の材料及び方法を使用して早発的染色体凝縮を誘導するインストラクションを含んでいることが好ましい。特に、該インストラクションは、有糸分裂促進剤で細胞又は細胞群を刺激する必要なく、細胞又は細胞群で早発的染色体凝縮を誘導させるための詳細なプロトコールを提供するものである。

【0053】

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染色体スプレッドの調製及び分析

PCCスプレッドは、必要な処理の直後に、細胞遺伝学的手法に従い調製することができる。簡潔には、低張性塩化カリウム（0.075M）溶液で細胞を5分間にわたって処理して、酢酸：メタノール（1：3）固定液で固定する。固定された細胞は、酸洗浄されたガラス板上に落される。

【0054】

スプレッド染色体を直接的に可視化するために、スライドを染色する。適当な染色剤が当業者には既知であり、例えば、光学顕微鏡による観察には、ギームザ染色剤の4%水溶液を使うことができる。1000倍の拡大率でコード化されたスライドを分析することができる。染色体の少なくとも部分的分離を示している凝縮した染色質物質を持つ細胞は、PCCスプレッドとしてスコア化される。 10

【0055】

PCCインデックスは、以下のように決定される。

$$PCC \text{ スプレッド数} / (\text{分裂間期細胞数} + PCC \text{ スプレッド数}) \times 100$$

蛍光インシットハイブリダイゼーション分析（FISH）を伴う実験については、染色体スプレッドの調製後、全染色体DNAハイブリダイゼーションプローブは、一つ以上の染色体に特異的である。任意的に、全染色体DNAハイブリダイゼーションプローブは、検出可能な成分で直接にラベル化され、スプレッド染色体の分析に使用される。このようなラベル化された染色体プローブは、市販されている。例えば、スペクトラム緑色蛍光色素でラベル化された染色体1に特異的な全染色体プローブは、Vysis Incから得られる。 20

【0056】

インシットハイブリダイゼーション及び染色体ペインティング（chromosome painting）は、本技術分野で周知の技法（例えば、Brown et al. (1992) Int. J. Radiat. Oncol. Biol. Phys. 24, 279-286）を使って行われる。

【0057】

以下に開示された本発明の実施例では、Vysis Incからの染色体1プローブが、製造者のプロトコールに従って使われた。他の適当なプローブは、当業者に既知であり、本発明の意図から逸脱することなく使用され得る。他の好ましいプローブには、病的状態に特異的なプローブが含まれる。 30

【0058】

DAPI及びフルオレセインイソチオシアネート（FITC）用のフィルターが装備された蛍光顕微鏡（Leitz）下で染色体1異常を分析するために、4,6ジアミジノ2フェニールインドール（DAPI）を含む培地に細胞をマウントすることができる。

【0059】

コード化されたスライドは、染色体1を伴う異常を分析するために、1000倍の拡大率で観察することができる。染色体異常分析は、以下の一般的基準に基づく。

【0060】

分析においてインキュベートされる細胞は、次のうちの一つ以上（好ましくは全て）を示すべきである：（a）DAPI対比染色によって決定される凝縮された染色質物質を持つ染色体の少なくとも部分的分離、（b）明るい緑色蛍光シグナルを持つ、2つ以上のはっきりと分離された染色体1特異的スポット（シグナル緑色スポットを持つ細胞は、オーバーラップシグナルで起こるので、含めなかった）、（c）蛍光強度が類似のスポット、及び（d）偽処理されたコントロールからのサンプルで観察されたスポットの約15から100%の領域を表す領域。 40

【0061】

コントロールサンプルのスポット領域は、常に均一ではない。それは、染色体凝縮の異なりからであり、少しの場合には、顕微鏡下での角度表象（angular presentation）による。このような曖昧な場合には、分析から細胞は除外されるべきである。 50

【0062】

本発明の範囲又は本発明の実施形態から逸脱することなく他の適当な修飾及び適用が本発明の材料及び方法に成され得ることは当業者には容易に認識されよう。本発明を詳細に記載してきた。本発明は、図面及び以下の非限定的な実施例を参照することにより、一層明らかに理解されよう。

【0063】

実施例

(実施例1)

有糸分裂促進剤刺激細胞における早発的染色体凝縮の誘導

比較目的のため及びホスファターゼ阻害剤の適当なレベルを決定するために、先行技術の方法を使用して、HPBLsにおいて早発的染色体凝縮を誘導させた。 10

【0064】

エネルギー源で補充された細胞培地中で、上述のようにして調製したHPBLsをインキュベートする。PCCのための最適OA濃度及びインキュベーション期間を決定するために、フィットヘマグルチニン(PHA、 $10\mu\text{g/ml}$; Murex Diagnostics)を培地に引き続き加えて、増殖を刺激した。この完全培地は、有糸分裂増強因子を含まなかった。

【0065】

OAを単独で含む細胞培地中での未刺激HPBLのインキュベーションでは、PCC誘導は起こらなかった。従って、PHAは、細胞周期進行を助けるために使われた。 $100\mu\text{M}$ のATPを含む細胞培地中、 $0.25\sim 1\mu\text{M}$ の範囲の濃度のOAでHPBLを処理し、24時間までの種々の期間にわたって37でインキュベートした。上述の説明のように、スライドを調製してPCCインデックスを決定した。 20

【0066】

図2Aは、ギムザで染色された有糸分裂促進剤刺激HPBLにおいて、OAで処理することによって誘導されたPCCを示す代表的な写真である。細胞膜の溶解、染色質物質の凝縮、及び染色体の部分的分離は、OA誘導PCCを特徴付けた。分離されていない染色体は、分裂中期染色体又は有糸分裂細胞融合法によって誘導されたPCCと比較すると、より凝縮されていないように見え、染色体凝集塊がほとんどの細胞において依然として見える。 30

【0067】

図3は、有糸分裂促進剤刺激HPBLモデルにおける、OA濃度及びインキュベーション期間のPCC誘導に対する効果を示す。二つ以上の独立の実験からプールされたデータが示されている。各濃度及び時間の点は、1000個を超える細胞を代表する。有糸分裂促進剤刺激HPBLをOA($0.25\mu\text{M}$)で処理することにより、1時間以内で、コントロールと比較して、PCCインデックスで決定される有意のPCCレベル($p < 0.001$ 、学生のt検定)が得られた。PCCインデックスは、 $1\mu\text{M}$ 濃度で8時間で最大61%に達した。 $0.75\mu\text{M}$ 濃度では、インデックスは、2時間でピークであり、20%の細胞がPCCを提示し、24時間にわたるまでこのレベルに維持された。 $0.75\mu\text{M}$ 濃度でのOAは、細胞増殖抑制性でなく、有糸分裂促進剤刺激HPBLも出る適度に高いPCC収率を誘導すると考えられる。従って、未刺激HPBLにおいてPCCを誘導するための、 $p34^{cdc2}$ /サイクリンBキナーゼを用いた更なる研究は、この濃度を用いて行った。 40

【0068】

OA又はカリクリンA等のホスファターゼ阻害剤を用いて有糸分裂促進剤刺激HPBLを処理することにより、染色質物質が早発的に誘導されることについては、以前に実証されている。これらの研究においては、HPBLは、PHA刺激の41時間後から45時間後に、1時間から6時間の種々の期間にわたって、 $0.1\mu\text{M}$ から $0.5\mu\text{M}$ のOA用量(Gotoh et al. (1996) Int. J. Radiat. Biol. 70, 517-520; Kanada et al. (1999) Int. J. Radiat. Biol. 75, 441-446)又は $0.05\mu\text{M}$ のカリクリンA(Durante et al. (19 50

98) *Int. J. Radiat. Biol.* 74, 457-462) で、細胞周期の S 期又は G₂ 期から有糸分裂様状態への進出を誘導するために処理された。本実験では、HPBL の有糸分裂促進剤刺激の直後から 24 時間後に処理された、0.25 μM から 1 μM の間の OA 濃度の効果を研究した。細胞周期進行の活性化を援助するために PHA を使った。この研究においては、早ければ 1 時間で、PCC 収率の有意な ($P < 0.01$) 上昇が観察された。これは、有糸分裂促進剤刺激 HPBL 集団の迅速に分化しない群では、DNA 複製前に PCC 誘導されることを示唆する。OA での処理後に早ければ 1 時間で観察された PCC インデックスの有意な ($P < 0.01$) 上昇は、他の研究による増殖細胞で見られるものに相当する (Gotoh et al. (1996) *Int. J. Radiat. Biol.* 70, 517-520; Durante et al. (1998) *Int. J. Radiat. Biol.* 74, 457-462; Coco-Martin et al. (1997) *Int. J. Radiat. Biol.* 71, 265-273; and Ghosh et al. (1992) *Exp. Cell Res.* 201, 535-540)。

【0069】

最適化研究では (図 3)、0.75 μM の OA により、2 時間で 20% のピーク PCC レベルが得られ、24 時間にわたるまでこのレベルに維持された。この用量は、未刺激 HPBL モデルにおいて PCC を誘導するための、p34^{cdc2} / サイクリン B キナーゼを用いた処理に使われた。この用量を選択したのは、PCC 収率だけでなく、PCC スプレッドの質にもよる。Kanada (Kanada et al. (1999) *Int. J. Radiat. Biol.* 75, 441-446) の観察と同じく、より高濃度の OA での持続性の処理では、スプレッドの質が悪いことが観察され、これはおそらく毒性によると思われる。更に、OA は、ヒト骨髄性の白血病の細胞株において、濃度依存及び時間依存により、細胞周期進行を止めることが分かった (Ishida et al. (1992) *J. Cell. Physiol.* 150, 484-492)。PCC 誘導可能な高濃度 (0.5 μM よりも高濃度) では、細胞周期停止は、G₁ - S 期に起こる。しかし、低濃度では、細胞周期停止は、G₂ - M 期に起こる (Ishida et al. (1992) *J. Cell. Physiol.* 150, 484-492)。

【0070】

(実施例 2)

休止細胞における早発的染色体凝縮の誘導

本発明の以下の実施例では、OA (0.75 μM) を含む ATP (100 μM) で補充された完全培地に p34^{cdc2} / サイクリン B キナーゼを加え、37℃ で 3 時間にわたってインキュベーションすることによって、未刺激 HPBL での PCC 誘導を達成した。2 つ以上の独立した実験から PCC インデックスを決定した。各データ点は、1000 個以上の細胞を代表する。プールされたデータを、有糸分裂促進剤刺激 HPBL モデルにおいて、OA での単独処理によって得られた収率と比較した。得られた結果を、先行例の先行技術の方法によって得られた結果と比較した。

【0071】

5 ユニット / ml まで低い濃度で p34^{cdc2} / サイクリン B キナーゼが存在することにより、未刺激 HPBL において PCC 誘導が起こった。この濃度では、PCC 収率は、有糸分裂促進剤刺激 HPBL において OA 単独により処理された群の収率よりも約 30% 高かった (図 4)。酵素濃度の増加により、濃度依存性で且つ有意な ($p < 0.05$; 学生の t 検定) 増加が PCC 収率に起こった (図 4)。また、染色質物質の凝集及びスプレッドが改善され、PCC スプレッドのより質の高いものがもたらされた (図 2B)。

【0072】

(実施例 3)

未刺激細胞からの染色体スプレッドを使用した放射線量の決定

未刺激細胞から調製された PCC スプレッドは、Coco-Martin 及び Beeg (Coco-Martin et al. (1997) *Int. J. Radiat. Biol.* 71, 265-273) によって記載された「スポットアッセイ」によって全染色体プローブでハイブリダイズされた特異的な染色体を伴う放射線誘導染色体異常の検出に適切であった。

【0073】

カリオマックス (Karyomax) 中の細胞懸濁液を 15 ml ポリプロピレン遠心分離管の中に

入れ、室温で、 60°C 施設の左右対称場において 1 Gy /分の線量率でガンマ線に晒した。照射源及び線量測定手法は、先に記載されている(Stankus et al. (1995) Int. Radiat. Biol. 68, 1-9)。線量率は、照射の前に組織等価イオン化チャンバで測定された。左右対称場は、2%の範囲内で均一である。放射線量-応答の研究では、未刺激HPBLを、PCC誘導前に、完全培地中で照射後21時間にわたって37でインキュベートした。

【0074】

OA、ATP及び $p34^{cdc2}$ /サイクリンBキナーゼを含む培地中で未刺激HPBLをインキュベートすることによって得られたPCCスプレッドにおいて、染色体1を伴う照射誘導による構造上の異常を持つ細胞を定量化するためにFISHを使った。この研究は、生物学的な線量測定への「スポットアッセイ」の潜在適応性について評価し、0から 7.5 Gy のガンマ線量への暴露に続く37での24時間修復インキュベーションを含んだ。上記で説明したように、PCCスプレッドを調製し、FISH手法を適用した。実験ごとの最大差異は重大ではなかった(1次自由度について、カイ自乗値 $=0.265$ 、 $p=0.606$)ので、4つの独立した実験からデータをプールした。各線量レベルは、2つ以上の実験を代表するものである。染色体1を伴っている異常を数えるために、少なくとも1000個の細胞を分析した。

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【0075】

照射されなかった細胞では、2つの蛍光(緑色)スポットが見られた。これは、染色体1の2つのコピーと考えられる(図2C)。照射された細胞では、染色体1に異常が誘導されたことにより、2つ以上の蛍光スポットが度々見られた(図2D)。これは、照射により誘導された断片又は置換であるだろうと考えられる。異なる線量のガンマ照射に晒された後での、染色体1を伴う異常を持つ細胞の頻度分布についてのデータについては、表1に示してある。

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【0076】

これらのデータにより、異常染色体1を持つ細胞は、 0 Gy から 7.5 Gy の間の放射線量と伴に増加することが実証される。これは、一般的に、細胞遺伝子学エンドポイントについての線量-効果とよく一致する。染色体1の過剰スポット数は、 0.5 Gy で細胞当たり 0.035 ± 0.0058 から 7.5 Gy で 0.236 ± 0.0126 へと、放射線量と併に増加した。FISH-ペイントPCCスプレッドにおける染色体1異常を持つ細胞のベースライン頻度は、 0.006 ± 0.0020 であった。2つのスポットを持つ細胞の頻度は、 0.5 Gy で 0.965 から 7.5 Gy で 0.803 へと減少し、2つを超えるスポットを持つ細胞の頻度に相当する増加があった(表)。染色体1について2つを超えるスポットを持つ細胞数は、 0 Gy から 7.5 Gy への放射線量に伴って増加し、 19.70 ± 1.258 パーセントで最大に達した(図5)。

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【0077】

異常染色体1を持つ数々の細胞の線量-反応データは、2つのモデルに適合した。荷重付加最小自乗回帰方法(荷重は平均値二乗のSEの逆数)によって適合する線形モデル($Y=(2.77 \pm 0.230)D + 0.90 \pm 0.431$ 及び $r^2=0.966$)及び非線形パワーモデル($Y=(5.70 \pm 0.46)D^{(0.61 \pm 0.05)}$ 及び $r^2=0.9901$)である。非線形パワーモデルと適合する場合、線量-反応曲線の横軸方向への曲がり観察された。

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【0078】

【表1】

オカダ酸及びp34^{cdc2}サイクリンBキナーゼ^aを用いた処置によって誘発されたPCCで可視化された、異なるガンマ線量に暴露された後の、異常型染色体1を持つ細胞の頻度分布解析

放射線量 (Gy)	全細胞数	染色体1のスポット数を持つ 細胞の頻度			>2スポットを持つ 細胞の頻度 平均±SE	過剰スポット数 ／細胞 平均±SE
		2スポット	3スポット	4スポット		
0.0	1500	0.994	0.006	—	0.006±0.0020	0.006±0.0020
0.5	1000	0.965	0.035	—	0.035±0.0058	0.035±0.0058
1.5	1000	0.917	0.083	—	0.083±0.0087	0.083±0.0087
3.0	1003	0.890	0.1096	—	0.110±0.0099	0.110±0.0099
4.5	1486	0.869	0.1232	0.008	0.131±0.8760	0.139±0.0088
6.0	1666	0.828	0.1477	0.024	0.172±0.0092	0.196±0.0092
7.5	1000	0.803	0.158	0.039	0.197±0.0126	0.236±0.0126

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^a データは、4つの独立した実験からプールされ、各線量レベルは2以上の実験を代表する。

【0079】

線量 - 反応の関係は、他の分裂中期-スプレッドに基づく細胞遺伝学アッセイ又は小核アッセイ (micronucleus assay) よりも、広範な線量範囲を持つ。非線形パワーモデル適合では、線量 - 反応曲線の横軸方向への下向き曲率が観察された。ただ1つの染色体対のみがペイントされており、これはゲノムの断片のみを表すので、放射線量を増加させるとシグナルの若干の飽和が起こると予期される。この効果は、高い放射線量では特に確かである。高い放射線量では、複合体 (置換物及び断片の両方) によって作られる数々の分離シグナルが制限される。所与の細胞においては核領域は一定である。更に、細胞当たりの平均値置換 (mean exchanges) は、低LET放射による正の上方向曲率で増加することが知られている。この場合、この曲率は、幾分小さくなる。屈曲をゆがめる (異なる線量 - 反応屈曲を持つ) 断片の含有のためである。非線形パワーモデルへのより良い適合により、このアッセイは比較的放射線量において感度が高いことが示唆される。このデータは、Coco-Martin及びBeggs (Coco-Martin et al. (1997) Int. J. Radiat. Biol. 71, 265-273) の先のデータで、OAによって誘導されたG₁期PCCにおけるヒト腺癌細胞株 (A549) をガンマ線照射することによって誘導された染色体4異常の測定を伴うものとよく一致する。

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【0080】

(実施例4)

放射線量の決定のインビボ検証

本明細書に記載された方法は、被検体が受ける放射線量を評価するために使用できる。このことは、異なる線量のガンマ線への暴露に続き、37°Cで24時間にわたり修復インキュベーションを行った後、HPBLsの早発的染色体凝縮スプレッドを使って実証された。このアッセイでは、未刺激HPBLにおいて、染色体1異常を伴う細胞当たりのベースライン頻度は、0.006±0.0020と観察された。この頻度は、他の細胞遺伝学アッセイについてのベースライン頻度よりも高い (例えば、分裂中期スプレッドではジセントリクス (細胞当たり0.001) が測定される)。より高いベースライン頻度は、一般には、異常を持っている若干の細胞が有糸分裂前に細胞集団から消失するので、分裂中期スプレッドをベースにした細胞遺伝学アッセイによっては検出されないことを示唆する。従って、本発明の方法は、細胞の状態をより正確に評価することができる。有糸分裂を経ることのできない細胞が依然としてデータセットに表示されて消失されないからである。

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【0081】

タイのバンコクで起こっている放射能漏れ源であるくず鉄からの⁶⁰Coガンマ線放射に晒された個人からHPBLサンプルを収集した。これらの個人は、0.1Gyから16Gyの放射線量を、200µSv/hに及び線量速度で受けていた。放射線に晒されたこれらの人 (30人以上) から、照射から約4ヶ月後に、12サンプルを収集し、コントロールを備えた9サンプルを先に記載したFISH法によって分析して、染色体1中の染色体異常の数について決定した。図6にデータを示す。図6は、放射線に照射された患者から単離された細胞においては、2つ以上の蛍光スポットを持つ細胞の割合が、正常コント

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ロール細胞と比較した場合に増加することを示している。

【0082】

照射された個々人のHPBLについて実行されたPCCアッセイによって例示されたように、本発明の方法は、生物線量測定のための直接的且つ高感度のツールを提供する (Pantelias et al. (1985) Mutat. Res. 149, 67-72; Prasanna et al. (1997) Health Phys. 72, 594-600; and Cornforth et al. (1983) Science 222, 1141-1143)。このアッセイは、吸収された線量を素早く (実験室で血液サンプルを受けてから24時間以内に) 予測することができ、効果的な臨床学的処置を可能にする。未刺激細胞について行われ、細胞分裂を要求しないので、放射誘導による細胞周期遅延 (Poncelet et al. (1988) Strehla nther. Und Onkol. 164, 542-543) 及び細胞死 (MacVittie et al. (1996) Advances in the Treatment of Radiation Injury, Elsevier Science, 263-269) 等の交絡因子 (cofounding factors) は、線量の見積もりと干渉しあわない。

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【0083】

これらの結果により、本発明の方法が、放射線暴露の生物学的線量測定につき、現在使われている手法 (有糸分裂細胞融合の後の、分裂中期又はPCCスプレッドの染色体異常の分析等) に比べて、より簡易で且つより信頼できる手法を提供できることが示される。本発明の方法は、未刺激細胞にPCCを誘導させ、特定の染色体に関与する異常を分析することを伴う。本方法は、早発的染色体凝縮を誘導させるために、有糸分裂促進因子を含み且つホスファターゼ阻害剤及びエネルギー源 (例えば、 $p34^{cdc2}$ / サイクリンBキナーゼ、OA及びATP) を任意に含む細胞培地中で試験細胞を単にインキュベートすることを伴い、簡易であり、代替的PCC誘導プロトコール (Pantelias et al. (1983) Somatic Cell Genet. 9, 533-547; Johnson et al. (1970) Nature 226, 717-722) と関連する技術的な専門知識を必要としない。

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【0084】

(実施例5)

卵母細胞、胚盤胞、幹細胞と胚細胞での染色体完全性の検査

実施例2の方法を使って、卵母細胞等の単一細胞、芽細胞からの極体または細胞、多発性細胞 (例えば、羊水サンプル又は確立されたヒト幹細胞株からの細胞) でPCCを誘導させた。マウスからの卵母細胞又は胚細胞を使うこともできる。細胞又は細胞 (複数) を、実施例2に記載された完全培地中で、37°Cで3時間にわたってインキュベートする。染色体スプレッドを調製して、先に記載された方法のいずれかを使って検査する。構造上の異常 (例えば、2つを超える蛍光スポット) は、FISH法を使って示されるか、染色体に結合する遺伝子座特定のプローブの欠損によって示される。健全な胚または細胞株を、培養中又はインキュテロで維持し、健全な卵母細胞 (その相当する極体は検証される) は受精させる。異常な細胞は培養中で維持されず、以降の手順にも使用されない。

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【0085】

完全培地の最適化のためには、複数の細胞を含むサンプルを部分に分け、実施例2の完全培地中で各部分をインキュベートするが、各部分には、ホスファターゼ阻害剤 (オカダ酸又はカリクリンA) 又はエネルギー源 (ATP) 又はサイクリンキナーゼ ($p34^{cdc2}$ / サイクリンBキナーゼ) が異なる濃度で含まれている。利用可能なサンプル部分の数に従って、複数の成分を最適化することができる。染色体スプレッドを得るためには、37°Cで3時間経った後細胞を収穫し、低張性処理して、メタノール/酢酸で固定し、スライド上に置き染色する。PCCが誘導される細胞の割合を、各サンプルについて計算し、線量-反応の関係を決定する。その後、一つ以上の成分の最適な濃度を使って、引き続き分析のための完全培地を調製する。

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【0086】

単一細胞胚又は卵母細胞を操作するためには、マイクロ操作法を使う。マイクロピペット・チップに細胞を付けて保持し、完全培地を有する培養皿に入れる。PCCの誘導前に、37°Cで数時間にわたって細胞をインキュベートする。代替として、 $p34^{cdc2}$ / サイクリンBキナーゼと、オカダ酸又はカリクリンAのいずれかとの溶液を、マイクロインジ

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エクシオン又は電気泳動により、細胞に導入する。その後、培養皿の中身を、低張液、固定液と続けて置換して、染色体スプレッドを調製する。第二の代替として、インキュベーション用の完全培地を含む毛細管内に細胞を保持して、吸引と補充により先に記述した処理を行う。この手順は、立体顕微鏡の下で行われる。同様にして染色体スプレッドを調製する。

【0087】

上述の通り、インシツハイブリダイゼーション、染色体ペインティング又は蛍光顕微鏡により染色体を検査する。染色体が市販の蛍光分子でラベルされる全染色体DNAハイブリダイゼーションは、単一の染色体に特異的である。インシツハイブリダイゼーション及び染色体ペインティングは、標準法に従い実施される。PCC誘導の後、DAPI及びFITC用のフィルターを備えた蛍光顕微鏡の下で、DAPI含有培地に細胞サンプルを置く。染色体1で研究されたような染色体異常が可視化され、種類と数について分析することができる。

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【0088】

(実施例6)

PCC感受性リンパ球部分母集団の高スループット単離

多数のサンプルを伴う細胞遺伝学的適用及び分析のためには、PCCの影響を受け易いリンパ球の部分母集団を単離する高スループット手順が必要である。現在の手順は、長たらく能率が悪い。つまり、密度勾配上(例えば、フィコール、Histopaque)での単離には、有糸分裂促進剤及びPHAを用いた処理が続けて行われる。その後、分裂中期スプレッドが調製され、コルセミドでの処理によって周期が止められる。その後、これらの細胞を培養して、有糸分裂収率が4-5%の部分母集団が生産される。

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【0089】

十分な数のPCC感受性末梢血液リンパ球を迅速に且つ簡易に生産するために、遠心分離管(例えば、50mlの円錐形の遠心分離管)中で、ロゼットセップ(Rosette Sep^(R))(Stem Cell Technologies)多価抗体を含むカクテルと全血液を混合させる。遠心分離管を室温にて20分間にわたりインキュベートする。有糸分裂促進剤及びPCC非感受性リンパ球及び非リンパ球白血球を抗体で架橋させて、四量体「ロゼット」複合体(tetrameric "rosette" complexes)を形成する。各遠心分離管中の内容物の下に、その後、フィコールが置かれ、20分間にわたって遠心にかける。上部の血漿層と下部のフィコール層との間に、PCC感受性である精製されたリンパ球部分母集団を含むインターフェースが形成される。不必要な白血球、赤血球及び他の細胞様及び粒状の血液成分は、底にペレットされる。

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【0090】

この手順は、多数の血液サンプルを含むためにスケラブルであり(自動化単離システムの使用により、1ラン当たり>500)、有糸分裂収率の10倍増加が達成可能である。その結果、この手順は、細胞遺伝学適用のための現在の方法にとって好ましい。免疫系疾患に関連する臨床学的適応に対して、この手順は、CD3+T細胞、CD4+T細胞及びCD8+T細胞等のT細胞部分母集団の単離のために、より良く適している。

【0091】

PCC感受性リンパ球部分母集団の単離もまた、ステムセップ(Stem Sep^(R))(Stem Cell Technologies)免疫磁気細胞選択アッセイを使って達成される。このアッセイでは、試薬カクテルは、サンプル中の不必要な細胞の表面に存在するマーカーに対しての抗体から成る。これらの抗体でラベルされた細胞は、磁気カラムを通過することにより効果的に除去される。その一方で、所望の細胞は、流れて、ラベル化されないで、高度に濃縮されて、カラムに集められる。Stem Sep^(R)免疫磁気陰性細胞選択は、メモリーCD4+T細胞(CD4+T細胞カクテルにCD45RAを追加)、静止CD4+T細胞(CD4+T細胞カクテルの一つ以上のCD25, CD69HLA-DRを追加)、静止CD8+T細胞(CD8+T細胞カクテルの一つ以上のCD25, CD27, CD69HLA-DRを追加)、T細胞(T細胞カクテルにT

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C R を追加)及び T 細胞 (T 細胞カクテルに T C R を追加)の単離に使われる。

【0092】

本発明の明瞭な理解のために、例示及び実施例として、その詳細につき十分に記載してきた。当業者には、条件、調剤及び他のパラメタについて広範囲かつ等価の範囲内で、本発明を修飾又は置換したとしても、本発明の範囲又は特定の実施形態に影響を与えることなく、同一のことが実行されることが明らかであろう。これらの修飾又は置換は、請求項の範囲内であると意図される。

【0093】

本明細書で言及された全ての出版物、特許及び特許出願は、本発明と関連する当業者の水準を示すものであり、参照として本明細書に明確に取り込まれている。 10

【図面の簡単な説明】

【0094】

【図1】種々の細胞周期における種々の有糸分裂増強因子のアセンブリ及びリン酸化状態を表す図解である。

【図2】(A)から(D)は、早発性染色体凝縮を誘導するために処理した細胞の染色体スプレッドを示す。(A)は、ギームザ染色されたHPBLsの染色体スプレッドの顕微鏡写真であり、早発性染色体凝縮は、有糸分裂促進剤刺激及びOA存在下でのインキュベーションによって誘導されている。(B)は、ギームザ染色されたHPBLsの染色体スプレッドの顕微鏡写真であり、早発性染色体凝縮は、p34^{cdc2}/サイクリンBキナーゼ及びOAの存在下でのインキュベーションによって誘導されている。(C)は、非照射HPBLsの染色体1のFISH分析を示す顕微鏡写真であり、早発性染色体凝縮は、p34^{cdc2}/サイクリンBキナーゼ及びOAの存在下でのインキュベーションによって誘導されている。(D)は、照射HPBLsの染色体1のFISH分析を示す顕微鏡写真であり、早発性染色体凝縮は、p34^{cdc2}/サイクリンBキナーゼ及びOAの存在下でのインキュベーションによって誘導されている。 20

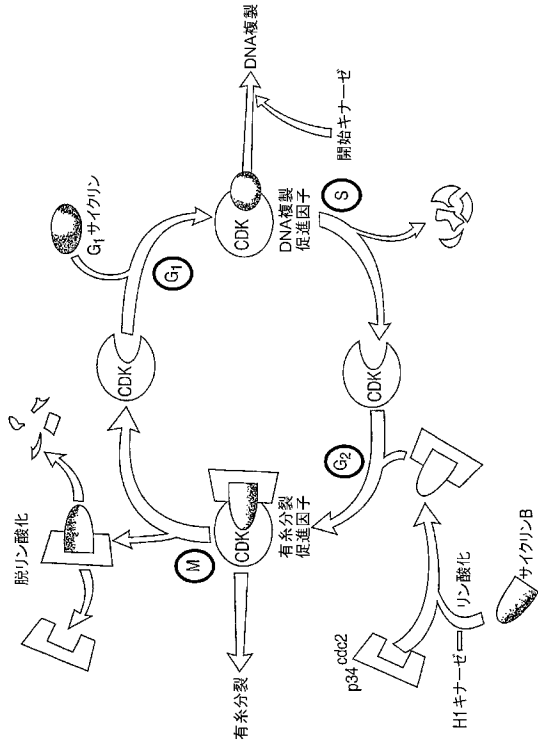
【図3】種々のインキュベーション時間及びOA濃度が、有糸分裂促進剤刺激HPBLsのPCCインデックスに及ぼす効果を示す図である。

【図4】種々のp34^{cdc2}/サイクリンBキナーゼ濃度が、p34^{cdc2}/サイクリンBキナーゼ処理HPBLsのPCCインデックスに及ぼす効果を示す図である。 30

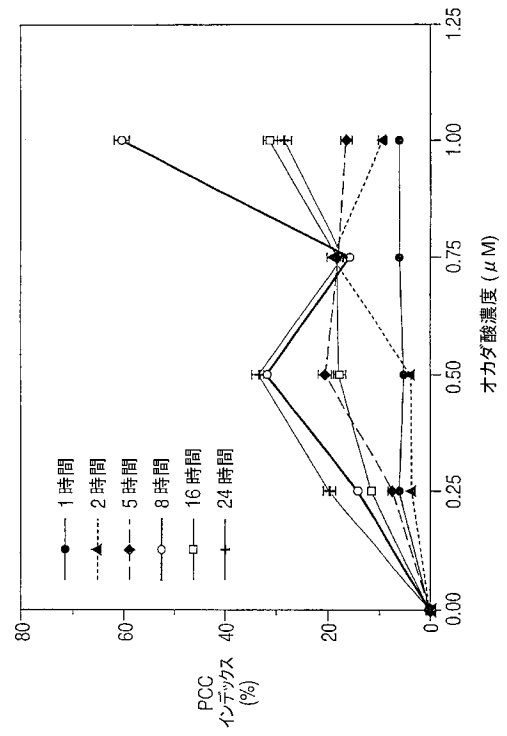
【図5】放射線で誘導された染色体異常を持つ細胞についての線量-反応曲線を示す図である。

【図6】放射線に晒された患者から単離された細胞において、2つ以上の蛍光スポットを持つ細胞の割合が、正常コントロール細胞と比較して増加していることを示す図である。

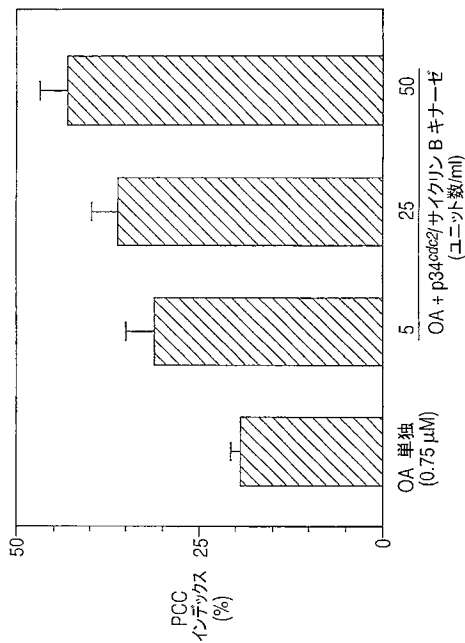
【 図 1 】



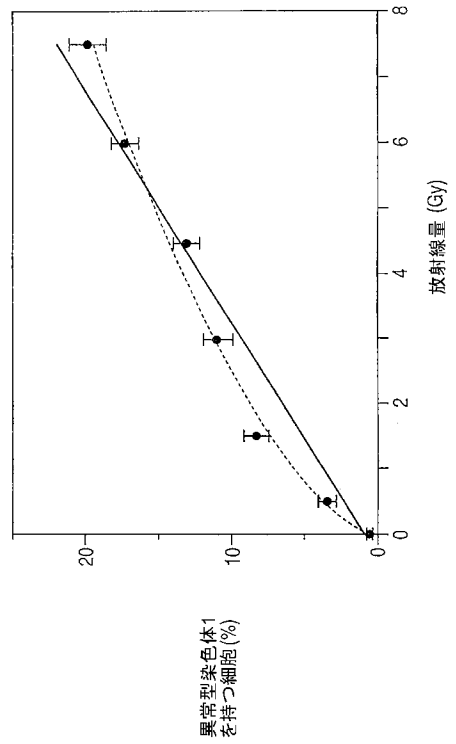
【 図 3 】



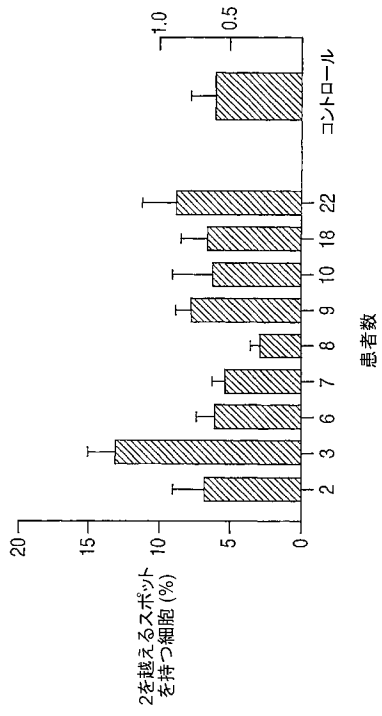
【 図 4 】



【 図 5 】



【 図 6 】



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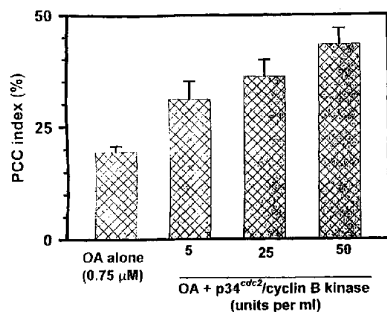
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(54) Title: MATERIALS AND METHODS FOR THE INDUCTION OF PRIMATURE CHROMOSOME CONDENSATION



(57) Abstract: The present invention provides a simple and rapid method to study chromosome aberrations using unstimulated cells. Premature chromosome condensation (PCC) is induced by incubating unstimulated cells in the presence of a mitosis-enhancing factor. The present method is more rapid than the prior art methods involving stimulating cells or fusion with mitotic cells. The condensed chromosomes produced by the present methods can be used in numerous forms of cytogenetic analysis, in particular, with *in situ* hybridization probes and chromosome painting. This technique can be applied to biological dosimetry of radiation exposures involving uniform whole-body low-LET (linear energy transfer) exposures.

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Materials and Methods for the Induction of Premature Chromosome Condensation**Inventors**

Pataje G.S. Prasanna and William F. Blakely

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Field of the Invention

The present invention concerns the fields of cytogenetics, molecular cytogenetic, cell biology, genetic toxicology and genomics. In particular, the present invention concerns methods of inducing premature chromosome condensation and methods of analyzing genetic material using the condensed chromosomes.

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Background

Various environmental insults have the potential to induce physical damage to genetic material. In addition to exposure to environment toxins, accidental exposure of human beings to radiation is a major concern. Development of simple and rapid methods is required for

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insult dose assessment, which will benefit the treatment of exposed individuals. Muller and Streffer (Muller *et al.* (1991) *Int. J. Radiat. Biol.* 59, 863-873) published a comprehensive review of biological indicators of radiation damage, explaining current techniques of biological dosimetry for radiation dose assessment. After exposure to high

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doses of radiation, sufficient numbers of mitotic cells are not available for dose assessment by the routine metaphase spread chromosome aberration analysis. The premature chromosome condensation (PCC) assay, performed on an exposed individual's blood lymphocytes, is viewed as a rapid biodosimetry method of clinical significance (Pantelias *et al.* (1985) *Mutat. Res.* 149, 67-72; Blakely *et al.* (1995) *Stem Cells* 13, 223-230; and Prasanna *et al.* (1997) *Health Phys.* 72, 594-600. Currently, physical damage to chromosomes can be analyzed by observation of chromosomes after preparation of a metaphase spread. Chromosomes are visualized in mitotic cells following a short-term cell culture in which cells are stimulated into proliferation by a mitogen and then subjected to cell cycle arrest with colchicine or colcemid. The

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chromosomes are observed under a microscope after being treated either by staining or by hybridizing with a fluorescent probe. This technique depends upon the successful stimulation of the cells to proliferate and requires 48 hours or more of cell culture to obtain useful yields. The technique is labor intensive and requires experience in cytogenetic techniques to practice. The analysis is further complicated by cell killing and cell cycle delay induced by the treatment. In addition, the low yield of condensed chromosomes frequently requires large numbers of metaphase spreads to obtain statistically significant data.

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Another method of analyzing physical damage to chromosomes involves inducing the premature chromosome condensation (PCC) in the cells and preparing a chromosome spread. Historically, premature chromosome condensation was accomplished by fusing the cells of interest with mitotic cells. This resulted in the condensation of the chromosomes in the test cells into chromatid-like structures. Although this technique does produce premature chromosome condensation, there are several difficulties associated with its practice. The technique requires a constant supply of mitotic cells to be fused with the test cells. The culture and maintenance of the mitotic cells adds considerably to the expense of the method. Additionally, cell fusion techniques (for example, PEG mediated fusion) are inefficient and produce low and variable yields of fused cells. This results in a low and variable yield of premature chromosome condensation in the test cells (Pantelias *et al.* (1983) *Somatic Cell Genet.* 9, 533-547).

The deficiencies of mitotic cell fusion to induce premature chromosome condensation are well known in the art and the search for alternative simple and rapid protocols has been a topic of ongoing research (Gotoh *et al.* (1996) *Int. J. Radiat. Biol.* 70, 517-520; Kanda *et al.* (1999) *Int. J. Radiat. Biol.* 75, 441-446; Durante *et al.* (1998) *Int. J. Radiat. Biol.* 74, 457-462; and Coco-Martin *et al.* (1997) *Int. J. Radiat. Biol.* 71, 265-273). Recently, premature chromosome condensation has been induced by stimulating cells with a mitogen and then culturing the cells in the presence of phosphatase inhibitors. Inhibitors of type 1 and 2A protein phosphatases have been used to induce PCC in proliferating cells (Gotoh *et al.* (1996) *Int. J. Radiat. Biol.* 70, 517-520; Kanda *et al.* (1999) *Int. J. Radiat. Biol.* 75, 441-446; Durante *et al.* (1998) *Int. J. Radiat. Biol.* 74, 457-462; and Coco-Martin *et al.* (1997) *Int. J. Radiat. Biol.* 71, 265-273).

The condensed chromosomes prepared by phosphatase inhibitor treatment were evaluated for biological dosimetry applications using chromosome aberration analysis in PCC spreads. Premature chromosome condensation was induced by okadaic acid (OA) (Gotoh *et al.* (1996) *Int. J. Radiat. Biol.* 70, 517-520; Kanda *et al.* (1999) *Int. J. Radiat. Biol.* 75, 441-446) or calyculin A (Durante *et al.* (1998) *Int. J. Radiat. Biol.* 74, 457-462) in mitogen stimulated cells and obtained 48 hours after mitogen-stimulation. Durante *et al.* (Durante *et al.* (1998) *Int. J. Radiat. Biol.* 74, 457-462) demonstrated that simultaneous measurement of chromosome aberrations in G₁ and M phases is possible by using whole-chromosome probe fluorescence *in situ* hybridization (FISH) technique following exposure to 200-kVp x-rays. It has also been shown that incubation of actively dividing tumor cell lines in a cell culture medium containing OA or calyculin A results in PCC induction (Coco-Martin *et al.* (1997) *Int. J. Radiat. Biol.* 71, 265-273). Using whole-chromosome-specific probes, chemically

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induced PCC spreads containing radiation-induced chromosome aberrations are readily identified as cells with more than 2 chromosome spots. A difference in radiosensitivity was demonstrated between radiosensitive (SCC61) and radioresistant (A549) cell lines (Coco-Martin *et al.* (1997) *Int. J. Radiat. Biol.* 71, 265-273).

5 Although the use of phosphatase inhibitors produces premature chromosome condensation in stimulated or proliferating cells, presently available methods still require an incubation period in order to produce sufficiently high yields of premature chromosome condensation to be useful for chromosome aberration analysis.

10 **Brief Summary of the Invention**

Notwithstanding the methods discussed above, there exists a need in the art for rapid and simple methods to assess the damage of genetic material by environmental insults. Presently, a major cause of the difficulty in making such assessments is the time and labor required to generate condensed chromosomes for subsequent analysis. The present invention

15 meets this long felt need by providing a cell culture medium that induces premature chromosome condensation rapidly and in high yields in unstimulated cells. The present invention does away with the need for cell fusion to induce premature chromosome condensation in unstimulated cells and does away with the need for stimulation and subsequent incubation required by other methods known in the art. Condensed chromosomes
20 prepared using the materials and methods of the present invention have been used to demonstrate that damage to specific chromosomes in unstimulated HPBL can be studied by FISH with whole-chromosome-specific probes in chemically-induced PCC spreads. The methods of the present invention are simpler and faster than those known in the art and are particularly suited to automated, high throughput assays of chromosome damage. These
25 methods have numerous applications including rapid biological dosimetry applications.

The present invention provides a cell culture medium for inducing premature chromosome condensation in a cell. In preferred embodiments, the cell culture medium comprises one or more mitosis enhancing factors. In some embodiments, the mitosis enhancing factor may be one or more cyclins, cyclin kinases, histone kinases, cyclins,
30 topoisomerases, structural maintenance of chromosome (SMC) proteins, histones, *cdk1* substrate, and components of mitosis promoting factor. In a preferred embodiment, the mitosis enhancing factor is p34^{cdc2}/cyclin B kinase.

A cell culture medium of the present invention may comprise a phosphatase inhibitor. In such cases, the phosphatase inhibitor may include one or more of okadaic acid, salts of
35 okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin,

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dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR. A cell culture medium of the invention may comprise an energy source, preferably ATP and/or GTP.

5 The present invention provides a method of analyzing a chromosome by incubating a cell with a medium comprising a mitosis enhancing factor, wherein the medium induces premature chromosome condensation, and analyzing the condensed chromosome. In some
10 embodiments, the mitosis enhancing factor may be one or more of cyclin kinases, histone kinases, cyclins, topoisomerases, SMC proteins, cdk1 substrate, histones, and components of mitosis promoting factor (MPF). In some preferred embodiments, the mitosis enhancing factor may include p34^{cdc2}/cyclin B kinase.

15 A medium for use in the method of analyzing a chromosome may comprise a phosphatase inhibitor. Preferably, the phosphatase inhibitor may be one or more of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR. The medium may comprise an energy source, preferably, ATP and/or GTP. The medium may include a transfection reagent.

20 The method for analyzing a chromosome may be practiced on any type of cell. In some embodiments, the cell may be a lymphocyte. Preferably, the cell is a mammalian cell. In some embodiments, the cell is a human peripheral blood lymphocyte. In some
25 embodiments, the cell is a murine cell, preferably a murine peripheral blood lymphocyte.

The method of analyzing a chromosome may include preparing a chromosome spread. The method may include hybridizing one or more oligonucleotides to one or more
30 chromosomes and enumerating chromosome spots. In some embodiments, one or more of the oligonucleotides comprises a detectable moiety. Preferably, the detectable moiety is a fluorescent moiety although the detectable moiety may be one or more of biotin, digoxigenin, antigens, enzymes and haptens.

35 The present invention also provides a method of assessing clastogenicity of a compound by contacting a cell with the compound, incubating the cell with a medium comprising a mitosis enhancing factor, wherein the medium induces premature chromosome condensation and analyzing the condensed chromosomes for breakage, structural and/or numerical aberrations. In some embodiments, the cell is contacted with the medium and the compound simultaneously. In other embodiments, the cell may be contacted with the compound and then transferred to a suitable medium. It may be desirable in some instances to incubate the cell after contact with the compound for a period of time sufficient to allow

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chromosomal repair. In some embodiments, the mitosis enhancing factor may be one or more of cyclin kinases, histone kinases, cyclins, topoisomerases, SMC proteins, cdk1 substrate, histones, and components of mitosis promoting factor (MPF). In some preferred embodiments, the mitosis enhancing factor may include p34^{cdc2}/cyclin B kinase.

5 A medium for use in the method of assessing clastogenicity of a compound may comprise a phosphatase inhibitor. Preferably, the phosphatase inhibitor may be one or more of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and
10 microcystin-YR. The medium may comprise an energy source, preferably, ATP and/or GTP. The medium may include a transfection reagent.

The method of assessing clastogenicity of a compound may be practiced on any type of cell. In some embodiments, the cell may be a lymphocyte. Preferably, the cell is a mammalian cell. In some embodiments, the cell is a human peripheral blood lymphocyte. In
15 some embodiments, the cell is a murine cell, preferably a murine peripheral blood lymphocyte.

The method of assessing clastogenicity of a compound may include preparing a chromosome spread. The method may include hybridizing one or more oligonucleotides to one or more chromosomes and enumerating chromosome spots. In some embodiments, one
20 or more of the oligonucleotides comprises a detectable moiety. Preferably, the detectable moiety is a fluorescent moiety although the detectable moiety may be one or more of biotin, digoxigenin, antigens, enzymes and haptens.

The present invention also provides a method of assessing toxicity of a compound by contacting a cell with the compound, incubating the cell with a medium comprising a mitosis
25 enhancing factor, wherein the medium induces premature chromosome condensation and analyzing the condensed chromosomes. In some embodiments, the cell is contacted with the medium and the compound simultaneously. In other embodiments, the cell may be contacted with the compound and then transferred to a suitable medium. It may be desirable in some instances to incubate the cell after contact with the compound for a period of time sufficient to
30 allow chromosomal repair. In some embodiments, the mitosis enhancing factor may be one or more of cyclin kinases, histone kinases, cyclins, topoisomerases, SMC proteins, cdk1 substrate, histones, and components of mitosis promoting factor (MPF). In some preferred embodiments, the mitosis enhancing factor may include p34^{cdc2}/cyclin B kinase.

A medium for use in the method of assessing toxicity of a compound may comprise a
35 phosphatase inhibitor. Preferably, the phosphatase inhibitor may be one or more of okadaic

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acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR.

5 The medium may comprise an energy source, preferably, ATP and/or GTP. The medium may include a transfection reagent.

The method of assessing toxicity of a compound may be practiced on any type of cell. In some embodiments, the cell may be a lymphocyte. Preferably, the cell is a mammalian cell. In some embodiments, the cell is a human peripheral blood lymphocyte. In some

10 The method of assessing toxicity of a compound may include preparing a chromosome spread. The method may include hybridizing one or more oligonucleotides to one or more chromosomes and enumerating chromosome spots. In some embodiments, one or more of the oligonucleotides comprises a detectable moiety. Preferably, the detectable moiety is a fluorescent moiety although the detectable moiety may be one or more of biotin, digoxigenin, antigens, enzymes and haptens.

15 The present invention also provides a method of detecting chromosomal abnormalities in a subject by isolating one or more cells from the subject, incubating the cell with a medium comprising a mitosis enhancing factor, wherein the medium induces premature chromosome condensation and analyzing the condensed chromosomes for abnormalities. In some embodiments, the mitosis enhancing factor may be one or more of cyclin kinases, histone kinases, cyclins, topoisomerases, SMC proteins, cdk1 substrate, histones, and components of mitosis promoting factor (MPF). In some preferred

20 A medium for use in the method of detecting chromosomal abnormalities in a subject may comprise a phosphatase inhibitor. Preferably, the phosphatase inhibitor may be one or more of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR. The medium may comprise an energy source, preferably, ATP and/or GTP.

30 The medium may include a transfection reagent.

The method of detecting chromosomal abnormalities in a subject may be practiced on any type of cell. In some embodiments, the cell may be a lymphocyte. Preferably, the cell is a mammalian cell. In some embodiments, the cell is a human peripheral blood lymphocyte. In some embodiments, the cell is a murine cell, preferably a murine peripheral blood

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lymphocyte. In some embodiments, the cell may be obtained from a subject while the subject is *in utero*.

5 The method of detecting chromosomal abnormalities in a subject may include preparing a chromosome spread. The method may include hybridizing one or more oligonucleotides to one or more chromosomes and enumerating chromosome spots. In some
5 embodiments, one or more of the oligonucleotides comprises a detectable moiety. Preferably, the detectable moiety is a fluorescent moiety although the detectable moiety may be one or more of biotin, digoxigenin, antigens, enzymes and haptens.

10 The present invention also provides a method of assessing a radiation dose received by a subject by isolating one or more cells from the subject, contacting one or more cells with a medium comprising a mitosis enhancing factor, wherein the medium induces premature
10 chromosome condensation and analyzing the condensed chromosomes for abnormalities such as breakage, structural and/or numerical aberrations. In some embodiments, the mitosis enhancing factor may be one or more of cyclin kinases, histone kinases, cyclins, topoisomerases, SMC proteins, cdk1 substrate, histones, and components of mitosis
15 promoting factor (MPF). In some preferred embodiments, the mitosis enhancing factor may include p34^{cdc2}/cyclin B kinase.

A medium for use in the method of assessing a radiation dose received by a subject may comprise a phosphatase inhibitor. Preferably, the phosphatase inhibitor may be one or
20 more of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR. The medium may comprise an energy source, preferably, ATP and/or GTP. The medium may include a transfection reagent.

25 The method of assessing a radiation dose received by a subject may be practiced on any type of cell. In some embodiments, the cell may be a lymphocyte. Preferably, the cell is a mammalian cell. In some embodiments, the cell is a human peripheral blood lymphocyte. In some embodiments, the cell is a murine cell, preferably a murine peripheral blood
lymphocyte.

30 The method of assessing a radiation dose received by a subject may include preparing a chromosome spread. The method may include hybridizing one or more oligonucleotides to one or more chromosomes and enumerating chromosome spots. In some embodiments, one or more of the oligonucleotides comprises a detectable moiety. Preferably, the detectable
moiety is a fluorescent moiety although the detectable moiety may be one or more of biotin,
35 digoxigenin, antigens, enzymes and haptens.

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The present invention also provides a composition comprising a cell and a cell culture medium, wherein the cell culture medium comprises a mitosis enhancing factor and induces premature chromosome condensation in the cell. In the compositions of the present invention, the mitosis enhancing may be one or more of cyclin kinases, histone kinases, 5 cyclins, topoisomerases, structural maintenance of chromosome (SMC) proteins, histones, *cdk1* substrate, and components of mitosis promoting factor. In some preferred embodiments, the mitosis enhancing factor may be p34^{cdc2}/cyclin B kinase. The compositions of the present invention may include a phosphatase inhibitor. The phosphatase inhibitor may be one or more of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, 10 cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR. The compositions may also comprise an energy source, preferably ATP and/or GTP.

The present invention provides kits for the induction of premature chromosome 15 condensation in test cells. In some embodiments, the kits may comprise one or more containers of a cell culture medium which comprises a mitosis enhancing factor and induces premature chromosome condensation in the cell. The mitosis enhancing may be one or more of cyclin kinases, histone kinases, cyclins, topoisomerases, structural maintenance of chromosome (SMC) proteins, histones, *cdk1* substrate, and components of mitosis promoting 20 factor. In some preferred embodiments, the mitosis enhancing factor may be p34^{cdc2}/cyclin B kinase. The kits of the present invention may include one or more containers holding one or more phosphatase inhibitors. The phosphatase inhibitor may be one or more of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin- 25 LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR. The kits may also comprise one or more containers holding an energy source, preferably ATP and/or GTP. The kits of the present invention may comprise one or more containers holding one or more transfection reagents.

30 **Brief Description of the Drawings**

Figure 1 is a schematic representation of the assembly and phosphorylation state of various mitosis enhancing factors in various stages of the cell cycle.

Figures 2A-2D show chromosome spreads of cells treated to induce premature chromosome condensation. Figure 2A is a photomicrograph of a Giemsa stained 35 chromosome spread of HPBLs in which premature chromosome condensation was induced by

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- mitogen stimulation and incubation in the presence of OA. Figure 2B is a photomicrograph of a Giemsa stained chromosome spread of HPBLs in which premature chromosome condensation was induced by incubation in the presence of p34^{cdc2}/cyclin B kinase and OA. Figure 2C is a photomicrograph showing a FISH analysis of chromosome 1 in un-irradiated
- 5 HPBLs in which premature chromosome condensation was induced by incubation in the presence of p34^{cdc2}/cyclin B kinase and OA. Figure 2D is a photomicrograph showing a FISH analysis of chromosome 1 in irradiated HPBLs in which premature chromosome condensation was induced by incubation in the presence of p34^{cdc2}/cyclin B kinase and OA.
- 10 Figure 3 is a graph showing the effects of various incubation times and OA concentrations on PCC index in mitogen stimulated HPBLs.
- Figure 4 is a graph showing the effects of various p34^{cdc2}/cyclin B kinase concentrations on PCC index in p34^{cdc2}/cyclin B kinase treated HPBLs.
- Figure 5 is a graph showing the dose-response curve for cells with radiation induced chromosome aberrations.
- 15 Figure 6 is a graph showing the increase in the percentage of cells with two or more fluorescent spots in cells isolated from patients exposed to radiation when compared to normal control cells.

Detailed Description of the Invention

- 20 The present invention provides materials and methods for the induction of premature chromosome condensation in without the need to stimulate the cells with a mitogen. In addition, the present invention provides methods of analyzing genetic material by inducing premature chromosome condensation and analyzing the physical structure of the condensed chromosomes. The present invention is useful in any application requiring premature
- 25 chromosome condensation in a test cell. The invention is particularly useful in the fields of cytogenetics, molecular cytogenetics, cell biology, genetic toxicology and genomics.
- In some aspects, the present invention provides materials and methods useful in diagnostic cytogenetics. The materials and methods of the present invention may be used in prenatal, postnatal and pre-implantation testing to evaluate the genetic material of a test cell.
- 30 For example, the methods described herein may be used to evaluate the genetic material in a potential sperm donor to determine the presence or absence of chromosomal aberrations in the sperm. Likewise, the present invention may be used to analyze the genetic material of a subject while the subject is *in utero*.
- In some related aspects, the present invention can be used in cytogenetic research. In
- 35 the field of genomics, for example, the present invention may be used to detect genes

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associated with various syndromes characterized by chromosomal aberrations, for example Down's syndrome. In a particular embodiment, the present invention may be used to detect genes associated with microdeletion syndromes. In another embodiment, the present invention may be used to detect chromosomal anomalies (both numerical and structural) associated with cancer. In some preferred embodiments, the present invention may be used to detect gene amplification.

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In the field of environmental testing, the present invention may be used to assess the exposure of a subject to environmental insults. In some preferred embodiments, the present invention may be used to assess the radiation dose received by a subject. The radiation dose may have been received as a result of accidental exposure or may be the result of occupational exposure. The present invention may be particularly useful in cases of exposure of a large number of subjects as the capability of automating the present invention makes it well suited to a high throughput automated screening system. In other embodiments, the exposure of a subject to a compound which induces chromosomal abnormalities can be assessed.

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In some preferred embodiments, the present invention provides methods of assessing the toxicity of a drug. These methods are useful in the identification of potential chemotherapeutic agents where it is desirable to have an agent capable of inducing chromosomal breaks. In this aspect, the present methods may be used to assess the clastogenicity (ability to break chromosomes) of a particular agent. The present methods may also be used as an initial safety screen to determine whether a therapeutic agent induces chromosomal aberrations.

Cells

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Any type of cell having genetic material may be used in the practice of the present invention. For example, cells from heart, lung, liver, kidney, brain or other tissue may be used as a source of cells. The isolation of cells from various tissues may be accomplished using any technique known to those skilled in the art. In preferred embodiments, the cells are of mammalian origin, such as human or murine cells. In some preferred embodiments, peripheral blood lymphocytes may be used for premature chromosome condensation and analysis. In other preferred embodiments, cells may be oocytes or obtained from embryos, amniotic fluid or established cell lines, such as stem cell lines.

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The isolation of the cells to be used in the present invention may be by any means known to those skilled in the art. In some preferred embodiments, human peripheral blood lymphocytes (HPBLs) may be used. The isolation of peripheral blood lymphocytes is routine in the art. One suitable protocol is described below and other methods known to those skilled

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in the art could be used. In the following protocol, the peripheral blood lymphocytes were isolated from a human subject. They could equivalently be isolated from any subject. In some preferred embodiments, the subject may be mammalian. In other preferred embodiments, the subject may be a human or a mouse.

5 Lymphocytes may be isolated from whole blood samples using any suitable technique known to those skilled in the art. An example of a suitable technique is density gradient centrifugation, for example, using Histopaque 1077 (Sigma Chemical Co.). After centrifugation, cells may be collected and washed twice in phosphate-buffered saline (pH 7.0). The cells may then be re-suspended in a suitable cell culture medium. The selection of a suitable cell culture medium for a given type of cell is routine in the art. When the cells are 10 lymphocytes, a suitable medium may be Karyomax (Life Technologies Inc.). The cells may be re-suspended at a concentration suitable for subsequent analysis, for example, at a concentration of from about 1×10^6 cells per ml to about 1.5×10^8 cells per ml before use.

15 Cell Culture Media

The present invention provides a cell culture medium for inducing premature chromosome condensation in a test cell. Any suitable cell culture medium may be supplemented with one or more mitosis enhancing factors to be used as a cell culture medium of the invention. A suitable cell culture medium is one in which the cell of interest may be 20 maintained in a viable state throughout the duration the induction of premature chromosome condensation. Optionally, the suitable cell culture medium may be one in which the test cell may be maintained for a protracted period of time.

The cell culture media of the present invention will typically comprise various ingredients selected to maintain the viability of the test cells. Such ingredients include, but 25 are not limited to, amino acids, vitamins, inorganic salts, buffers or buffer salts, sugars, lipids, trace elements, cytokines and hormones. Suitable cell culture media are commercially available from, for example, Life Technologies Inc.

In preferred embodiments, a cell culture medium of the present invention will comprise one or more mitosis enhancing factors. Mitosis enhancing factors are agents 30 associated with the progression of the cell cycle into mitosis. Mitosis enhancing factors include, but are not limited to, cyclins, cyclin kinases, histone kinases, topoisomerases, SMC proteins, cdk1 substrate, histones, and components of mitosis promoting factor (MPF). In preferred embodiments, the mitosis enhancing factor may be a purified mitosis enhancing factor. The mitosis enhancing factor may be purified to any desired level of purity. 35 Preferably, the mitosis enhancing factor will at least 50% pure, *i.e.*, the mitosis enhancing

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factor will make up at least 50% by weight of a mitosis-enhancing-factor-containing material to be added to a culture medium. In other preferred embodiments, a mitosis enhancing factor may be 75% or greater pure, 80% or greater pure, 85% or greater pure, 90% or greater pure or 95% or greater pure. In a preferred embodiment, a cell culture medium of the present invention may comprise p34^{cdc2}/cyclin B kinase. Suitable p34^{cdc2}/cyclin B kinase is commercially available from, for example, New England Biolabs.

5 The mitosis enhancing factor may be added to the medium alone or in combination with other factors. The mitosis enhancing factor may be in the form of a native protein or a mutagenized protein. For example, fusion proteins comprising a mitosis enhancing factor may be used. A mitosis enhancing factor may be placed in frame with a protein or peptide portion of a different protein to produce a fusion protein. The construction of fusion proteins is routine in the art (see, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press). In preferred embodiments, the fusion proteins of the present invention may comprise, in addition to a mitosis enhancing factor, one or more ligands for a receptor to facilitate cellular uptake of the fusion protein, nuclear localization signals, purification tags, epitopes or the like. In a preferred embodiment, a cell culture medium of the present invention may comprise a fusion protein comprising a mitosis enhancing factor and a nuclear localization sequence. Suitable nuclear localization signals are known in the art and may be found, for example, in United States Patents 6,051,429 and 20 5,736,392.

In addition to mitosis enhancing factors, a cell culture medium of the present invention may comprise one or more energy sources including, but not limited to, ATP and GTP.

A cell culture medium of the present invention may optionally comprise one or more transfection reagents. As used herein, transfection reagent is seen to include any reagent which, when added to a cell culture medium, enhances the uptake by a test cell of a mitosis enhancing factor. Transfection reagents include, but are not limited to, neutral lipids, cationic lipids, mixtures of neutral and cationic lipids, proteins, peptides, lipoproteins, lipopeptides and the like. Suitable transfection reagents may be obtained commercially from, for example, 30 Promega Inc. and Life Technologies Inc. In some preferred embodiments, the transfection reagents of the present invention may comprise a peptide that enhances receptor mediated endocytosis. Examples of such transfection reagents may be found in United States Patent 6,103,529. The transfection reagent may be added directly to the media or may be combined with the mitosis enhancing factor prior to the addition of the mitosis enhancing factor to the 35 medium.

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A cell culture medium of the present invention may optionally comprise one or more phosphatase inhibitors. In some preferred embodiments, the protein phosphatases may specifically inhibit serine/threonine protein phosphatases. In some preferred embodiments the phosphatase inhibitors may specifically inhibit the protein phosphatases 1 and 2A. Suitable protein phosphatases include, but are not limited to, okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR.

10 Cell Culture Compositions

The cell culture media of the present invention may be used to formulate cell culture compositions comprising a cell or cell population and a culture medium of the invention. The cell may be any cell in which it is desired to induced premature chromosome condensation. Cells isolated from subjects are particularly preferred. The isolated cells may be derived from any organ or tissue in the subject including, but not limited to, blood, heart, lung, epithelial tissue and/or intestinal tissue.

Kits

The present invention contemplates kits adapted for use in cytogenetic research. Typically, the kits of the invention may comprise one or more containers holding a cell culture medium of the present invention. The cell culture medium may be in liquid form or in the form of a dry powder concentrate. The kits of the invention may comprise one or more containers holding one or more mitosis enhancing factors. The factors may be in solution or may be in the form of a dried powder. Kits of the invention may comprise one or more containers holding one or more phosphatase inhibitors. Optionally, kits of the invention may comprise one or more containers holding one or more transfection reagents and/or one or more energy sources which may be in solution or in dry form.

Kits of the present invention preferably comprise instructions for inducing premature chromosome condensation using the materials and methods of the present invention. In particular, the instructions may provide detailed protocols for inducing premature chromosome condensation in a cell or cell population without the need to stimulate the cell or cell population with a mitogen.

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Preparation and analysis of chromosome spreads

PCC spreads may be prepared according to standard cytogenetic procedures directly after the indicated treatment. Briefly, cells may be treated with a hypotonic potassium chloride (0.075 M) solution for 5 minutes and fixed in acetic: methanol (1:3) fixative. Fixed cells may be dropped onto acid cleaned glass slides.

To directly visualize the spread chromosomes, the slides may be stained. Suitable stains are known to those of skill in the art, for example, a 4% aqueous solution of Giemsa stain may be used for observation under a light microscope. Coded slides can be analyzed under 1000 × magnification. Cells with condensed chromatin material displaying at least partial separation of chromosomes are scored as PCC spreads.

The PCC index may be determined as follows.

$$\frac{\text{PCC spreads number}}{(\text{interphase cell number} + \text{PCC spreads number})} \times 100$$

For experiments involving fluorescent *in situ* hybridization analysis (FISH), after preparing a chromosome spread, whole-chromosome DNA hybridization probe specific for one or more chromosomes. Optionally, a whole chromosome DNA hybridization probe may be directly labeled with a detectable moiety and may be used to analyze the spread chromosomes. Such labeled chromosome-probes are commercially available. As an example, whole chromosome probe specific for chromosome 1 labeled with spectrum green fluorochrome may be obtained from Vysis Inc.

In situ hybridization and chromosome painting may be done using techniques well known in the art (see, for example, Brown *et al.* (1992) *Int. J. Radiat. Oncol. Biol. Phys.* 24, 279-286).

In the working example of the invention disclosed below, a chromosome 1 probe from Vysis was used according to the manufacturer's protocol. Other suitable probes are known to those skilled in the art and may be used without departing from the spirit of the invention. Other preferred probes include probes specific for pathological conditions.

Cells may be mounted in a medium containing 4,6-diamidino-2-phenyl-indole (DAPI) for analyzing chromosome 1 aberrations under a fluorescence microscope (Leitz) equipped with filters for DAPI and fluorescein isothiocyanate (FITC).

The coded slides may be observed at a 1000 × magnification for analyzing aberrations involving chromosome 1. Chromosome aberration analysis is based on the following general criteria:

The cells to be included in the analysis should show one or more (and preferably all) of the following: (a) at least partial separation of chromosomes with condensed chromatin

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material as determined by DAPI counterstain, (b) two or more clearly separated chromosome 1-specific spots with bright green fluorescent signals (cells with single green spots, arising because of overlapping signals, were not included), (c) spots that were similar in fluorescent intensity, and (d) an area representing about 15 to 100% of the area of the spots observed in samples from sham-treated controls.

The area of spots in the control samples may not always be uniform because of differential chromosome condensation and, in a few cases, angular presentation under the microscope. In such cases of ambiguity, cells should be excluded from analysis.

It will be readily apparent to those of skill in the art that other suitable modifications and adaptations may be made to the materials and methods of the present invention without departing from the scope of the invention or any embodiment thereof. Having now described the invention in detail, the invention may be more clearly understood with reference to the figures and the following non-limiting examples.

15 Examples

Example 1

Induction of premature chromosome condensation in mitogen stimulated cells

For purposes of comparison and in order to determine a suitable level of phosphatase inhibitor, premature chromosome condensation was induced in HPBLs using prior art methodology.

HPBLs prepared as described above may be incubated in cell culture medium supplemented with an energy source. In order to determine the optimal OA concentration and duration of incubation for PCC, phytohemagglutinin (PHA, 10 µg/ml; Murex Diagnostics) was subsequently added to the medium to stimulate proliferation. This complete medium did not contain a mitosis enhancing factor.

Incubation of unstimulated HPBL in a cell culture medium containing OA alone did not result in PCC induction, thus, PHA was used to help activate cell cycle progression. The HPBL were treated with OA at concentrations ranging from 0.25 to 1 µM in a cell culture medium containing 100 µM ATP and incubated at 37°C for varying durations of up to 24 hours. Slides were prepared and PCC index was determined as explained above.

Figure 2A is a representative photomicrograph showing PCC induced by a treatment with OA in a mitogen-stimulated HPBL stained with Giemsa. Dissolution of cell membrane, condensation of the chromatin material, and partial separation of chromosomes characterized OA-induced PCC. Undivided chromosomes appear less condensed compared to metaphase

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chromosomes or PCC induced by mitotic-cell fusion technique, and chromosome clumps are still visible in most cells.

Figure 3 shows the effect of OA concentration and duration of incubation on PCC induction in the mitogen-stimulated HPBL model. Pooled data is shown from two or more independent experiments with each concentration and time point representing more than 1,000 cells. Treatment of mitogen-stimulated HPBL with OA (0.25 μ M) resulted in significant ($p < 0.01$, Student's t-test) PCC levels determined by PCC index within 1 hour, compared with controls. The PCC index reached a maximum of 61% at a 1 μ M concentration at 8 hours. At a 0.75- μ M concentration, the index peaked at 2 hours, exhibiting PCC in about 20% of cells, and remained at that level for up to 24 hours. It appears that OA at 0.75 μ M concentration is not cytostatic and induces a reasonably high PCC yield in mitogen-stimulated HPBL model. Therefore, this concentration was used in further studies with p34^{cdc2}/cyclin B kinase to induce PCC in unstimulated HPBL.

It has been previously demonstrated that treatment of mitogen-stimulated HPBL with phosphatase inhibitors, such as OA or calyculin A, induces premature condensation of chromatin material. In those studies, HPBL were treated, 41 to 45 hours after PHA stimulation, with OA doses between 0.1 and 0.5 μ M (Gotoh *et al.* (1996) *Int. J. Radiat. Biol.* 70, 517-520; Kanda *et al.* (1999) *Int. J. Radiat. Biol.* 75, 441-446) or with 0.05 μ M calyculin A (Durante *et al.* (1998) *Int. J. Radiat. Biol.* 74, 457-462) for varying durations of 1 to 6 hours to induce entry into a mitosis-like state from the S- or G₂-phase of the cell cycle. In the present experiment, the effects of OA concentrations between 0.25 and 1 μ M, treated immediately and up to 24 hours after mitogen stimulation of HPBL were studied. PHA was used to help activate cell cycle progression. In this study, significant ($p < 0.01$) elevation in PCC yield was observed as early as 1 hour, indicating PCC induction before DNA replication in a rapidly dedifferentiating cohort of mitogen-stimulated HPBL population. The significant ($p < 0.01$) elevation in PCC index that was observed as early as 1 hour after treatment with OA is comparable to that seen in proliferating cells by others (Gotoh *et al.* (1996) *Int. J. Radiat. Biol.* 70, 517-520; Durante *et al.* (1998) *Int. J. Radiat. Biol.* 74, 457-462; Coco-Martin *et al.* (1997) *Int. J. Radiat. Biol.* 71, 265-273; and Ghosh *et al.* (1992) *Exp. Cell Res.* 201, 535-540).

In the optimization study (Figure 3), 0.75 μ M OA resulted in a peak PCC level of 20% at 2 hours and remained at that level for up to 24 hours. This dose was used for treatment with p34^{cdc2}/cyclin B kinase to induce PCC in the unstimulated HPBL model. Selection of this dose was based not only on PCC yield but also on quality of PCC spreads. Similar to the observations of Kanda *et al.* (Kanda *et al.* (1999) *Int. J. Radiat. Biol.* 75, 441-

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446), prolonged treatment with higher concentrations of OA was observed to result in poor spread quality, possibly due to toxicity. In addition, OA was found to arrest cell cycle progression in human myeloid leukemic cell lines in a concentration- and time-dependent manner (Ishida *et al.* (1992) *J. Cell. Physiol.* 150, 484-492). At higher PCC inducible
5 concentrations (above 0.5 μ M), cell-cycle arrest occurred at G₁-S-phase; but in lower concentrations cell-cycle arrest occurred at G₂-M phase (Ishida *et al.* (1992) *J. Cell. Physiol.* 150, 484-492).

Example 2

10 Induction of premature chromosome condensation in resting cells

In the following working example of the present invention, PCC induction in unstimulated HPBL was accomplished by the addition of p34^{cdc2}/cyclin B kinase to the complete media supplemented with ATP (100 μ M) containing OA (0.75 μ M) and incubation
15 for three hours at 37°C. PCC index was determined from two or more independent experiments, each data point representing more than 1,000 cells. The pooled data were compared with the yield obtained by OA treatment alone in the mitogen-stimulated HPBL model. The results obtained were compared to the results obtained using the prior art methodology of the preceding example.

The presence of p34^{cdc2}/cyclin B kinase at concentrations as low as 5 units per ml
20 resulted in PCC induction in unstimulated HPBL. At this concentration, the PCC yield was approximately 30% higher than the yield in the group treated with OA alone in mitogen-stimulated HPBL (Figure 4). An increase in the enzyme concentration resulted in a concentration-dependent and significant ($p < 0.05$; Student's t-test) increase in PCC yield (Figure 4). It also improved the spreading and condensation of the chromatin material,
25 yielding better quality PCC spreads (Figure 2B).

Example 3

Determination of radiation dosage using chromosome spreads from unstimulated cells

The PCC spreads prepared from unstimulated cells were suitable for detecting
30 radiation-induced chromosome aberrations involving a specific chromosome after hybridization with whole-chromosome probes by the "spot assay" described by Coco-Martin and Begg (Coco-Martin *et al.* (1997) *Int. J. Radiat. Biol.* 71, 265-273).

Cell suspension in Karyomax was placed in 15-ml polypropylene centrifuge tubes
and, at room temperature, was exposed to gamma rays at a dose rate of 1 Gy/min in a bilateral
35 field of a ⁶⁰Co facility. Radiation source and dosimetry procedures were previously described

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(Stankus *et al.* (1995) *Int. J. Radiat. Biol.* 68, 1-9). The dose rate was measured with a tissue-equivalent ionization chamber before irradiation. The field was uniform within 2%. In radiation dose-response studies, unstimulated HPBL were incubated at 37°C for 21 hours after exposure in complete medium before PCC induction.

5 FISH was used to quantify cells with radiation-induced structural aberrations involving chromosome 1 in PCC spreads obtained by incubating unstimulated HPBL in a medium containing OA, ATP, and p34^{cdc2}/cyclin B kinase. The study evaluated the potential application of this "spot assay" to biological dosimetry and included a 24 hour repair incubation at 37°C following exposure to gamma-ray doses of 0 to 7.5 Gy. PCC spreads were prepared and FISH technique was applied as explained above. Since the maximum difference between experiments was not significant (chi-square value = 0.265, p = 0.606 for one degree freedom), the data were pooled from four independent experiments, with each dose level representing two or more experiments. At least 1,000 cells were analyzed for enumerating aberrations involving chromosome 1.

15 In cells that had not been irradiated, two fluorescent (green) spots were seen, reflecting two copies of chromosome 1 (Figure 2C). Irradiated cells often exhibited more than two fluorescent spots (Figure 2D) due to induction of aberrations in chromosome 1, which likely reflect radiation-induced fragments or exchanges. The data on frequency distribution of cells with aberrations involving chromosome 1, after exposure to different doses of gamma radiation, are presented in Table 1.

20 These data demonstrate that the number of cells with aberrant chromosome 1 increases with radiation doses between 0 and 7.5 Gy. This, in general, is in good agreement with dose-effect increase for cytogenetic endpoints. The number of chromosome 1 excess spots increased with radiation dose from 0.035 ± 0.0058 per cell at 0.5 Gy to 0.236 ± 0.0126 at 7.5 Gy. Base-line frequency of cells with chromosome 1 aberrations in FISH-painted PCC spreads was 0.006 ± 0.0020 . Frequency of cells with two spots decreased from 0.965 at 0.5 Gy to 0.803 at 7.5 Gy with a corresponding increase in the frequency of cells with more than two spots (Table). The number of cells with more than two spots for chromosome 1 increased with radiation dose from 0 to 7.5 Gy and reached a maximum of 19.70 ± 1.258 per cent (Figure 5).

30 The dose-response data for the number of cells with aberrant chromosome 1 were fitted with two models, a linear model ($Y = (2.77 \pm 0.230)D + 0.90 \pm 0.431$ and $r^2 = 0.966$) fitted by the weighted least-squares regression method (weights were based on the reciprocal of the SE of the mean squared) and a nonlinear power model ($Y = (5.70 \pm 0.46)D^{(0.61 \pm 0.02)}$)

Table I
Frequency distribution analysis of cells with aberrant chromosome I after exposure to different doses of gamma rays as visualized in PCC induced by a treatment with okadaic acid and p34^{cdc2} cyclin B kinase^a

Radiation dose (Gy)	Total number of cells	Frequency of cells with number of chromosome I spots				Frequency of cells with > 2 spots		Number of excess spots/cell	
		2 spots	3 spots	4 spots	Mean ± SE	Mean ± SE	Mean ± SE		
0	1300	0.994	0.006	—	0.006 ± 0.0020	0.006 ± 0.0020	—	—	
0.5	1000	0.965	0.035	—	0.035 ± 0.0058	0.035 ± 0.0058	—	—	
1.5	1000	0.917	0.083	—	0.083 ± 0.0087	0.083 ± 0.0087	—	—	
3.0	1003	0.890	0.1096	—	0.110 ± 0.0099	0.110 ± 0.0099	—	—	
4.5	1486	0.869	0.1232	0.008	0.131 ± 0.8760	0.139 ± 0.0088	0.008	0.008	
6.0	1666	0.828	0.1477	0.0240	0.172 ± 0.0092	0.196 ± 0.0092	0.0240	0.0240	
7.5	1000	0.803	0.1580	0.0390	0.197 ± 0.0126	0.236 ± 0.0126	0.0390	0.0390	

^aData were pooled from four independent experiments with each dose level representing two or more experiments.

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and $r^2 = 0.9901$). When fitted with a nonlinear power model, a bending of the dose-response curve towards the abscissa was observed.

The dose-response relationship has a broader dose range than other metaphase-spread based cytogenetic assays or micronucleus assay. With the nonlinear power model fit, the observed downward curvature of the dose-response curve towards the abscissa. Since only one chromosome pair was painted, which represents only a fraction of the genome, some saturation of the signal was expected with increasing radiation dose. This effect is particularly true at higher radiation doses where the number of separate signals produced by complexes (both exchanges and fragments) is restricted, with nuclear area being constant for a given cell. In addition, mean exchanges per cell are known to increase with a positive upward curvature with low-LET radiation. In this case, this curvature was somewhat mitigated because of the inclusion of fragments (which have different dose-response curves) that distorted the curve. The better fit with a nonlinear power model suggests that this assay may be more sensitive at lower radiation doses. This data is in good agreement with earlier data of Coco-Martin and Begg (Coco-Martin *et al.* (1997) *Int. J. Radiat. Biol.* 71, 265-273), which involved a measurement of chromosome 4 aberrations induced by gamma irradiation in a human adenocarcinoma cell line (A549) in G₁-phase PCC induced by OA.

Example 4

20 *In vivo* validation of determination of radiation dosage

The methods disclosed herein can be used to assess the dose of radiation received by a subject. This was demonstrated using premature chromosome condensation spreads of HPBLs performed after a 24 hour repair incubation at 37°C following exposure to different doses of gamma rays. A base-line frequency of 0.006 ± 0.0020 per cell involving chromosome 1 aberrations was observed in unstimulated HPBL for this assay. This is higher than base-line frequencies for other cytogenetic assays (*e.g.*, dicentric (0.001 per cell) measured in metaphase spreads). A higher base-line frequency, in general, suggests that some cells carrying aberrations are lost from the cell population before mitosis and, therefore, are not detected by the metaphase-spread-based cytogenetic assays. Thus, the present methods more accurately assess the condition of the cells, since cells that are not competent to undergo mitosis are still represented in the data set and are not lost.

HPBL samples were collected from individuals who had been exposed to ⁶⁰Co gamma radiation from a scrap metal source, a radiation leak occurring in Bangkok, Thailand. These individuals received radiation doses of 0.1 to 16 Gy, at a dosage rate of up to 200 μSv/h. From those exposed to the radiation (over 30 people), twelve samples were collected

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approximately four months after exposure, and nine samples with controls were analyzed by the FISH method described above to determine the number of chromosomal aberrations in chromosome 1. These data are presented in Figure 6, which shows the increase in the percentage of cells with two or more fluorescent spots in cells isolated from patients exposed to radiation when compared to normal control cells.

The methods of the present invention, as exemplified by the PCC assay performed on HPBL of exposed individuals, provide a direct and sensitive cytogenetic tool for biodosimetry (Pantelias *et al.* (1985) *Mutat. Res.* 149, 67-72; Prasanna *et al.* (1997) *Health Phys.* 72, 594-600; and Cornforth *et al.* (1983) *Science* 222, 1141-1143). The assay can rapidly predict absorbed dose (within 24 hours of the receipt of a blood sample in the laboratory) to enable effective clinical treatment. Since it is conducted on unstimulated cells and does not require cell division, confounding factors such as radiation-induced cell-cycle delay (Poncelet *et al.* (1988) *Strehlanther. und Onkol.* 164, 542-543) and death (MacVittie *et al.* (1996) *Advances in the Treatment of Radiation Injury*, Elsevier Science, 263-269) do not interfere with dose estimates.

These results indicate that the present method provides a simpler and more reliable techniques for biological dosimetry of radiation exposures than currently used techniques such as analysis of chromosome aberrations in metaphase or PCC spreads after mitotic-cell fusion. The present method involves inducing PCC in unstimulated cells and analyzing aberrations involving specific chromosomes. This method, involving a simple incubation of test cells in a cell culture medium containing a mitosis enhancing factor and optionally a phosphatase inhibitor and an energy source (for example, p34^{cdc2}/cyclin B kinase, OA and ATP), to induce premature chromosome condensation, is simple and does not require the high degree of technical expertise associated with alternative PCC-inducing protocols (Pantelias *et al.* (1983) *Somatic Cell Genet.* 9, 533-547; Johnson *et al.* (1970) *Nature* 226, 717-722).

Example 5

Examination of chromosomal integrity in oocytes, blastocysts, stem cells and embryonic cells

Using the methods in Example 2, PCC is induced in a single cell, such as an oocyte, polar body or cell from a blastocyst, or multiple cells, such as an amniotic fluid sample or cells from an established human stem cell line. Oocytes or embryonic cells from mice can also be used. The cell or cells are incubated in the complete medium described in Example 2 for 3 hours at 37°C. Chromosome spreads are prepared, and the chromosomes are examined using any of the methods described on page 15. Structural abnormalities are indicated, *e.g.*, by more than 2 bright fluorescent spots, using the FISH technique, or by failure of a locus

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specific probe to bind to a chromosome. Healthy embryos or cell lines are maintained in culture or *in utero*, and healthy oocytes, whose corresponding polar bodies are tested, are fertilized. Abnormal cells are not maintained in culture or used in further procedures.

For optimization of the complete medium, samples containing multiple cells can be split into portions, each of which is incubated in the complete medium of Example 2, but in which each portion contains a different concentration of a phosphatase inhibitor (okadaic acid or calyculin A) or an energy source (ATP) or a cyclin kinase (p34^{cdc2}/cyclin B kinase). Multiple components can be optimized, according to the number of sample portions available. After three hours at 37°C, the cells are harvested, subjected to hypotonic treatment, fixed with methanol/acetic acid, placed on slides and stained to obtain chromosome spreads. The percentage of cells in which PCC is induced is calculated for each sample, and a dose-response relationship is determined. The optimal concentration of one or more components is then used to prepare complete medium for subsequent analyses.

Micromanipulation techniques are used to manipulate single-cell embryos or oocytes. The cell is held attached to a micropipette tip and contained in a culture dish with complete medium. The cell is incubated in medium for several hours at 37°C prior to induction of PCC. Alternatively, a solution of p34^{cdc2}/cyclin B kinase and either okadaic acid or calyculin A is introduced into the cell by microinjection or by electroporation. The contents of the dish are then replaced with, successively, hypotonic solution and fixative, and a chromosome spread is prepared. As a second alternative, the cell is held within a capillary tube containing complete medium for incubation, and the aforementioned treatments performed by aspiration and refilling. This procedure is carried out under a stereomicroscope. A chromosome spread is prepared in a similar fashion.

The chromosomes are examined by *in situ* hybridization, chromosome painting or fluorescence microscopy, as described above. Whole-chromosome DNA hybridization, in which the chromosome is labeled with a commercially available fluorochrome, is specific for single chromosomes. *In situ* hybridization and chromosome painting are carried out according to standard methods. Following PCC induction, the cell sample is mounted in medium containing DAPI under a fluorescence microscope equipped with filters for DAPI and FITC. Chromosome aberrations, such as those studied in chromosome 1, are visible and can be analyzed for type and number.

Example 6

High-throughput isolation of PCC-sensitive lymphocyte subpopulations

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For cytogenetic applications and analyses involving large numbers of samples, high-throughput procedures for isolating subpopulations of lymphocytes that are susceptible to PCC are required. Currently procedures are tedious and inefficient, *i.e.*, isolation on a density gradient (*e.g.*, Ficoll, Histopaque), followed by treatment with a mitogen and PHA.

5 Metaphase spreads are then prepared, and the cycle arrested by treatment with colcemid. These cells are then cultured, all to produce a subpopulation with a mitotic yield of 4-5%.

To quickly and simply produce adequate numbers of PCC-sensitive peripheral blood lymphocytes, whole blood is mixed with a cocktail containing RosetteSep[®] (Stem Cell Technologies) multivalent antibodies in centrifuge tubes, *e.g.*, 50 ml conical centrifuge tubes.

10 The tubes are incubated for 20 minutes at room temperature. Mitogen and PCC-insensitive lymphocytes and non-lymphocytic white blood cells are cross-linked by the antibodies to form tetrameric "rosette" complexes. The contents in each tube are then underlaid with Ficoll, and the tubes are spun for 20 minutes. An interface containing a purified lymphocyte subpopulation that is PCC-sensitive is formed between an upper plasma layer and a lower
15 Ficoll layer. Unwanted white blood cells, red cells and other cellular and particulate blood components are pelleted to the bottom.

This procedure is scalable to include a large number of blood samples (>500 per run using an automated isolation system), and a ten-fold increase mitotic yield is achievable. As a result, this procedure is preferable to current methods for cytogenetic applications. For

20 clinical applications related to immune system disorders, this procedure is well-suited for the isolation of T cell subpopulations such as CD3+ T cells, CD4+ T cells and CD8+ T cells.

Isolation of PCC-sensitive lymphocyte subpopulations is also accomplished using StemSep[®] (Stem Cell Technologies) immunomagnetic cell selection assay. In this assay, the reagent cocktails consist of antibodies directed against markers present on the surface of the
25 unwanted cells in the sample. The cells labeled by these antibodies are efficiently removed by passage through a magnetic column, while the desired cells are collected in the column flow through, unlabeled and highly enriched. StemSep[®] immunomagnetic negative cell

selection is used for isolation of memory CD4+ T cells (CD4+ T cell cocktail plus CD45 RA),
Resting CD4+ T cell (CD4+ T cell cocktail plus one or more of CD25, CD69, HLA-DR),
30 Resting CD8+ T cell (CD8+ T cell cocktail plus one or more of CD25, CD27, CD69, HLA-DR), $\alpha\beta$ T cell (T cell cocktail plus TCR $\gamma\delta$) and $\gamma\delta$ T cell (T cell cocktail plus TCR $\alpha\beta$).

Having fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide
35 and equivalent range of conditions, formulations and other parameters without affecting the

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scope of the invention or any specific embodiment thereof. Any such modifications or changes are intended to be within the scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains and
5 are specifically incorporated herein by reference.

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What is claimed:

1. A culture medium for inducing premature chromosome condensation in a cell comprising a cyclin kinase having mitosis enhancing properties, wherein the cyclin kinase is present in an amount effective to induce premature chromosome condensation.
5
2. A medium according to claim 1, wherein the cyclin kinase is p34^{cdc2}/cyclin B kinase.
- 10 3. A medium according to claim 1, further comprising a phosphatase inhibitor.
4. A medium according to claim 3, wherein the phosphatase inhibitor is selected from a group consisting of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endofthal, fenvalerate, 15 fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR.
5. A medium according to claim 1, further comprising an energy source.
20 6. A medium according to claim 5, wherein the energy source is selected from a group consisting of ATP and GTP.
7. A medium according to claim 1, further comprising a transfection reagent.
25 8. A kit comprising the medium of any one of claims 1-7.
9. A method of analyzing a chromosome, comprising:
(a) incubating a cell with a medium comprising a cyclin kinase having mitosis enhancing properties, wherein the cyclin kinase is present in an amount effective to induce 30 premature chromosome condensation; and
(b) analyzing the condensed chromosome.
10. A method of assessing clastogenicity of a compound, comprising:
(a) contacting a cell with the compound;

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(b) incubating the cell with a medium comprising a cyclin kinase having mitosis enhancing properties, wherein the cyclin kinase is present in an amount effective to induce premature chromosome condensation; and

5 (c) analyzing the condensed chromosomes for breakage, structural and/or numerical aberrations.

11. A method according to claim 10, wherein the cell is contacted with the medium and the compound simultaneously.

10 12. A method according to claim 10, further comprising incubating the cell after contact with the compound for a period of time sufficient to allow chromosomal repair.

13. A method of assessing toxicity of a compound, comprising:

(a) contacting a cell with the compound;

15 (b) incubating the cell with a medium comprising a cyclin kinase having mitosis enhancing properties, wherein the cyclin kinase is present in an amount effective to induce premature chromosome condensation; and

(c) analyzing the condensed chromosomes.

20 14. A method according to claim 13, wherein the cell is contacted with the medium and the compound simultaneously.

15. A method according to claim 13, further comprising incubating the cell after contact with the compound for a period of time sufficient to allow chromosomal repair.

25 16. A method of detecting chromosomal abnormalities in a subject, comprising:

(a) isolating one or more cells from the subject;

(b) contacting at least one cell with a medium comprising a cyclin kinase having mitosis enhancing properties, wherein the cyclin kinase is present in an amount effective to

30 induce premature chromosome condensation; and

(c) analyzing the condensed chromosomes for chromosome abnormalities.

17. A method according to claim 16, wherein chromosomal abnormalities are analyzed based on an evaluation of the number of condensed chromosome domains or spots

35 within a cell.

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18. A method according to claim 17, wherein the subject is *in utero*.
19. A method according to claim 17, wherein the abnormality is a numerical
5 abnormality.
20. A method according to claim 17, wherein the abnormality is a structural abnormality.
- 10 21. A method of assessing a radiation dose received by a subject, comprising:
(a) isolating one or more cells from the subject;
(b) contacting at least one cell with a medium comprising a cyclin kinase having mitosis enhancing properties, wherein the cyclin kinase is present in an amount effective to induce premature chromosome condensation; and
15 (c) analyzing the condensed chromosomes for chromosome abnormalities.
22. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the cyclin kinase is p34^{cdc2}/cyclin B kinase.
- 20 23. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the medium further comprises a phosphatase inhibitor.
24. A method according to claim 23, wherein the phosphatase inhibitor is selected from a group consisting of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid,
25 cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR.
25. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the
30 medium further comprises an energy source.
26. A method according to claim 25, wherein the energy source is selected from a group consisting of ATP and GTP.

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27. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the medium further comprises a transfection reagent.
28. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the cell is a lymphocyte.
5
29. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the cell is a mammalian cell.
30. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the cell is a human peripheral blood lymphocyte.
10
31. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the cell is a murine cell.
15
32. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the cell is a murine peripheral blood lymphocyte.
33. A method according to any one of claims 9, 10, 13, 16 or 21, wherein analyzing the chromosome comprises preparing a chromosome spread.
20
34. A method according to any one of claims 9, 10, 13, 16 or 21, wherein analyzing the chromosomes comprises hybridizing an oligonucleotide to at least one or more chromosomes and enumerating chromosome spots.
25
35. A method according to claim 34, wherein the oligonucleotide comprises a detectable moiety.
36. A method according to claim 35, wherein the detectable moiety is a fluorescent moiety.
30
37. A method according to claim 35, wherein the detectable moiety is selected from a group consisting of biotin, digoxigenin, antigens, enzymes and haptens.

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38. A composition comprising a cell and a cell culture medium comprising a cyclin kinase having mitosis enhancing properties, wherein the cyclin kinase is present in an amount effective to induce premature chromosome condensation.
- 5 39. A composition according to claim 38, wherein the cyclin kinase is p34^{cdc2}/cyclin B kinase.
40. A composition according to claim 38, further comprising a phosphatase inhibitor.
- 10 41. A composition according to claim 40, wherein the phosphatase inhibitor is selected from a group consisting of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR.
- 15 42. A composition according to claim 38, further comprising an energy source.
43. A composition according to claim 42, wherein the energy source is selected from a group consisting of ATP and GTP.
- 20 44. A composition according to claim 38, further comprising a transfection reagent.
45. A kit comprising the composition of any one of claims 38-44.

FIGURE 1

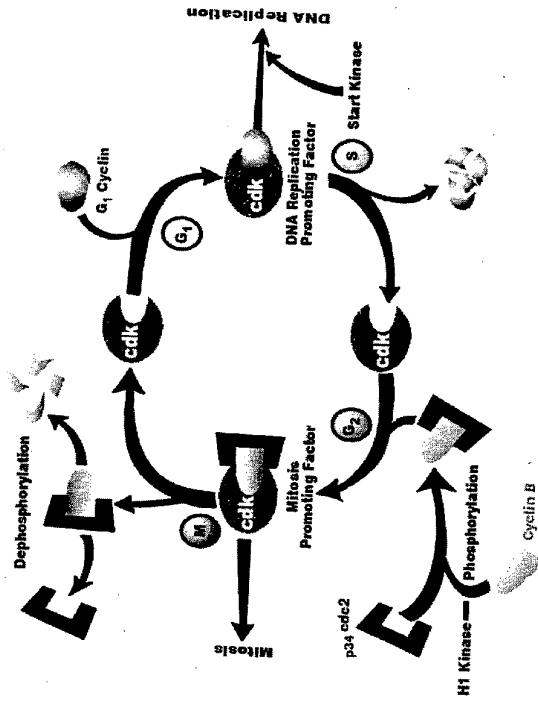


FIGURE 2

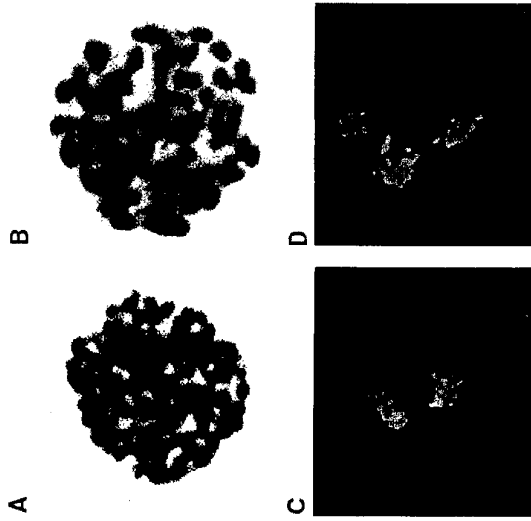


FIGURE 3

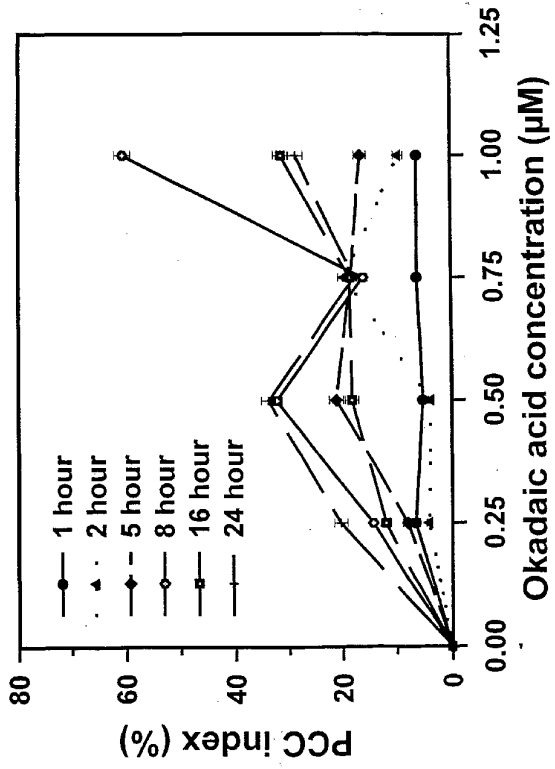


FIGURE 4

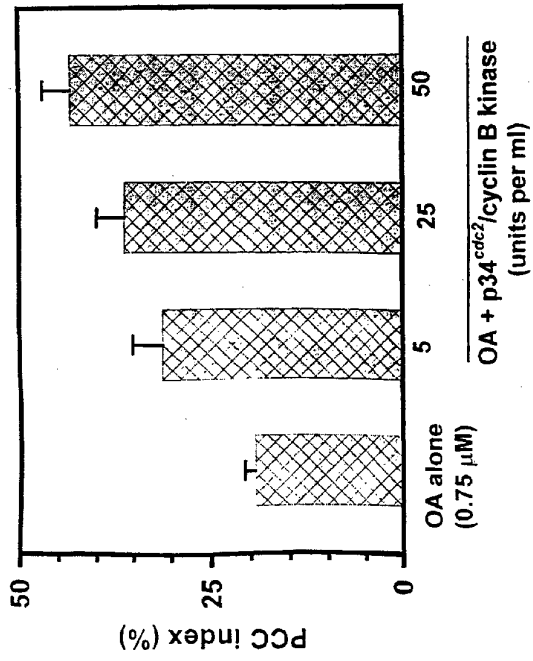
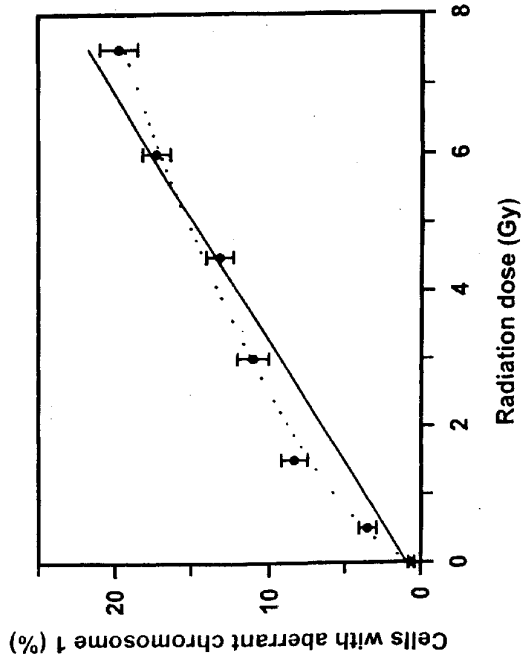


FIGURE 5

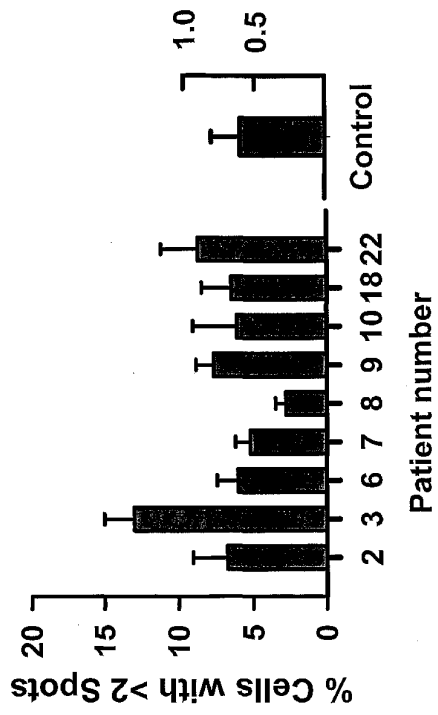


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Figure 6



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(54) Title: MATERIALS AND METHODS FOR THE INDUCTION OF PREMATURE CHROMOSOME CONDENSATION

(57) Abstract: The present invention provides a simple and rapid method to study chromosome aberrations using unstimulated cells. Premature chromosome condensation is induced by incubating unstimulated cells in the presence of a mitosis-enhancing factor. The condensed chromosomes produced by the present methods can be used in numerous forms of cytogenetic analysis, in particular, with in situ hybridization probes and chromosome painting. This technique can be applied to biological dosimetry of radiation exposures involving uniform whole-body low-linear energy transfer exposures.

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(54) Title: MATERIALS AND METHODS FOR THE INDUCTION OF PREMATURE CHROMOSOME CONDENSATION

(57) Abstract: The present invention provides a simple and rapid method to study chromosome aberrations using unstimulated cells. Premature chromosome condensation is induced by incubating unstimulated cells in the presence of a mitosis-enhancing factor. The condensed chromosomes produced by the present methods can be used in numerous forms of cytogenetic analysis, in particular, with in situ hybridization probes and chromosome painting. This technique can be applied to biological dosimetry of radiation exposures involving uniform whole-body low-linear energy transfer exposures.

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Materials and Methods for the Induction of Premature Chromosome Condensation**Inventors**

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Field of the Invention

The present invention concerns the fields of cytogenetics, molecular cytogenetic, cell biology, genetic toxicology and genomics. In particular, the present invention concerns methods of inducing premature chromosome condensation and methods of analyzing genetic material using the condensed chromosomes.

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Background

Various environmental insults have the potential to induce physical damage to genetic material. In addition to exposure to environment toxins, accidental exposure of human beings to radiation is a major concern. Development of simple and rapid methods is required for

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insult dose assessment, which will benefit the treatment of exposed individuals.

Muller and Streffer (Muller *et al.* (1991) *Int. J. Radiat. Biol.* 59, 863-873) published a comprehensive review of biological indicators of radiation damage, explaining current techniques of biological dosimetry for radiation dose assessment. After exposure to high

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doses of radiation, sufficient numbers of mitotic cells are not available for dose assessment by the routine metaphase spread chromosome aberration analysis. The premature chromosome condensation (PCC) assay, performed on an exposed individual's blood lymphocytes, is viewed as a rapid biodosimetry method of clinical significance (Pantelias *et al.* (1985) *Mutat. Res.* 149, 67-72; Blakely *et al.* (1995) *Stem Cells* 13, 223-230; and Prasanna *et al.* (1997)

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Health Phys. 72, 594-600.

Currently, physical damage to chromosomes can be analyzed by observation of chromosomes after preparation of a metaphase spread. Chromosomes are visualized in mitotic cells following a short-term cell culture in which cells are stimulated into proliferation by a mitogen and then subjected to cell cycle arrest with colchicine or colcemid. The

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chromosomes are observed under a microscope after being treated either by staining or by hybridizing with a fluorescent probe. This technique depends upon the successful stimulation of the cells to proliferate and requires 48 hours or more of cell culture to obtain useful yields. The technique is labor intensive and requires experience in cytogenetic techniques to practice. The analysis is further complicated by cell killing and cell cycle delay induced by the

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treatment. In addition, the low yield of condensed chromosomes frequently requires large numbers of metaphase spreads to obtain statistically significant data.

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Another method of analyzing physical damage to chromosomes involves inducing the premature chromosome condensation (PCC) in the cells and preparing a chromosome spread. Historically, premature chromosome condensation was accomplished by fusing the cells of interest with mitotic cells. This resulted in the condensation of the chromosomes in the test cells into chromatid-like structures. Although this technique does produce premature chromosome condensation, there are several difficulties associated with its practice. The technique requires a constant supply of mitotic cells to be fused with the test cells. The culture and maintenance of the mitotic cells adds considerably to the expense of the method. Additionally, cell fusion techniques (for example, PEG mediated fusion) are inefficient and produce low and variable yields of fused cells. This results in a low and variable yield of premature chromosome condensation in the test cells (Pantelias *et al.* (1983) *Somatic Cell Genet.* 9, 533-547).

The deficiencies of mitotic cell fusion to induce premature chromosome condensation are well known in the art and the search for alternative simple and rapid protocols has been a topic of ongoing research (Gotoh *et al.* (1996) *Int. J. Radiat. Biol.* 70, 517-520; Kanda *et al.* (1999) *Int. J. Radiat. Biol.* 75, 441-446; Durante *et al.* (1998) *Int. J. Radiat. Biol.* 74, 457-462; and Coco-Martin *et al.* (1997) *Int. J. Radiat. Biol.* 71, 265-273). Recently, premature chromosome condensation has been induced by stimulating cells with a mitogen and then culturing the cells in the presence of phosphatase inhibitors. Inhibitors of type 1 and 2A protein phosphatases have been used to induce PCC in proliferating cells (Gotoh *et al.* (1996) *Int. J. Radiat. Biol.* 70, 517-520; Kanda *et al.* (1999) *Int. J. Radiat. Biol.* 75, 441-446; Durante *et al.* (1998) *Int. J. Radiat. Biol.* 74, 457-462; and Coco-Martin *et al.* (1997) *Int. J. Radiat. Biol.* 71, 265-273).

The condensed chromosomes prepared by phosphatase inhibitor treatment were evaluated for biological dosimetry applications using chromosome aberration analysis in PCC spreads. Premature chromosome condensation was induced by okadaic acid (OA) (Gotoh *et al.* (1996) *Int. J. Radiat. Biol.* 70, 517-520; Kanda *et al.* (1999) *Int. J. Radiat. Biol.* 75, 441-446) or calyculin A (Durante *et al.* (1998) *Int. J. Radiat. Biol.* 74, 457-462) in mitogen stimulated cells and obtained 48 hours after mitogen-stimulation. Durante *et al.* (Durante *et al.* (1998) *Int. J. Radiat. Biol.* 74, 457-462) demonstrated that simultaneous measurement of chromosome aberrations in G₁ and M phases is possible by using whole-chromosome probe fluorescence *in situ* hybridization (FISH) technique following exposure to 200-kVp x-rays. It has also been shown that incubation of actively dividing tumor cell lines in a cell culture medium containing OA or calyculin A results in PCC induction (Coco-Martin *et al.* (1997) *Int. J. Radiat. Biol.* 71, 265-273). Using whole-chromosome-specific probes, chemically

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induced PCC spreads containing radiation-induced chromosome aberrations are readily identified as cells with more than 2 chromosome spots. A difference in radiosensitivity was demonstrated between radiosensitive (SCC61) and radioresistant (A549) cell lines (Coco-Martin *et al.* (1997) *Int. J. Radiat. Biol.* 71, 265-273).

- 5 Although the use of phosphatase inhibitors produces premature chromosome condensation in stimulated or proliferating cells, presently available methods still require an incubation period in order to produce sufficiently high yields of premature chromosome condensation to be useful for chromosome aberration analysis.

10 **Brief Summary of the Invention**

Notwithstanding the methods discussed above, there exists a need in the art for rapid and simple methods to assess the damage of genetic material by environmental insults. Presently, a major cause of the difficulty in making such assessments is the time and labor required to generate condensed chromosomes for subsequent analysis. The present invention
15 meets this long felt need by providing a cell culture medium that induces premature chromosome condensation rapidly and in high yields in unstimulated cells. The present invention does away with the need for cell fusion to induce premature chromosome condensation in unstimulated cells and does away with the need for stimulation and subsequent incubation required by other methods known in the art. Condensed chromosomes
20 prepared using the materials and methods of the present invention have been used to demonstrate that damage to specific chromosomes in unstimulated HPBL can be studied by FISH with whole-chromosome-specific probes in chemically-induced PCC spreads. The methods of the present invention are simpler and faster than those known in the art and are particularly suited to automated, high throughput assays of chromosome damage. These
25 methods have numerous applications including rapid biological dosimetry applications.

The present invention provides a cell culture medium for inducing premature chromosome condensation in a cell. In preferred embodiments, the cell culture medium comprises one or more mitosis enhancing factors. In some embodiments, the mitosis
30 enhancing factor may be one or more cyclins, cyclin kinases, histone kinases, cyclins, topoisomerases, structural maintenance of chromosome (SMC) proteins, histones, *cdk1* substrate, and components of mitosis promoting factor. In a preferred embodiment, the mitosis enhancing factor is p34^{cdc2}/cyclin B kinase.

- A cell culture medium of the present invention may comprise a phosphatase inhibitor. In such cases, the phosphatase inhibitor may include one or more of okadaic acid, salts of
35 okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin,

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dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR. A cell culture medium of the invention may comprise an energy source, preferably ATP and/or GTP.

5 The present invention provides a method of analyzing a chromosome by incubating a cell with a medium comprising a mitosis enhancing factor, wherein the medium induces premature chromosome condensation, and analyzing the condensed chromosome. In some
10 embodiments, the mitosis enhancing factor may be one or more of cyclin kinases, histone kinases, cyclins, topoisomerases, SMC proteins, cdk1 substrate, histones, and components of mitosis promoting factor (MPF). In some preferred embodiments, the mitosis enhancing factor may include p34^{cdc2}/cyclin B kinase.

A medium for use in the method of analyzing a chromosome may comprise a phosphatase inhibitor. Preferably, the phosphatase inhibitor may be one or more of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA,
15 microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR. The medium may comprise an energy source, preferably, ATP and/or GTP. The medium may include a transfection reagent.

The method for analyzing a chromosome may be practiced on any type of cell. In some embodiments, the cell may be a lymphocyte. Preferably, the cell is a mammalian cell.
20 In some embodiments, the cell is a human peripheral blood lymphocyte. In some embodiments, the cell is a murine cell, preferably a murine peripheral blood lymphocyte.

The method of analyzing a chromosome may include preparing a chromosome spread. The method may include hybridizing one or more oligonucleotides to one or more chromosomes and enumerating chromosome spots. In some embodiments, one or more of the
25 oligonucleotides comprises a detectable moiety. Preferably, the detectable moiety is a fluorescent moiety although the detectable moiety may be one or more of biotin, digoxigenin, antigens, enzymes and haptens.

The present invention also provides a method of assessing clastogenicity of a compound by contacting a cell with the compound, incubating the cell with a medium
30 comprising a mitosis enhancing factor, wherein the medium induces premature chromosome condensation and analyzing the condensed chromosomes for breakage, structural and/or numerical aberrations. In some embodiments, the cell is contacted with the medium and the compound simultaneously. In other embodiments, the cell may be contacted with the compound and then transferred to a suitable medium. It may be desirable in some instances
35 to incubate the cell after contact with the compound for a period of time sufficient to allow

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- chromosomal repair. In some embodiments, the mitosis enhancing factor may be one or more of cyclin kinases, histone kinases, cyclins, topoisomerases, SMC proteins, cdk1 substrate, histones, and components of mitosis promoting factor (MPF). In some preferred embodiments, the mitosis enhancing factor may include p34^{cdc2}/cyclin B kinase.
- 5 A medium for use in the method of assessing clastogenicity of a compound may comprise a phosphatase inhibitor. Preferably, the phosphatase inhibitor may be one or more of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and
- 10 microcystin-YR. The medium may comprise an energy source, preferably, ATP and/or GTP. The medium may include a transfection reagent.
- The method of assessing clastogenicity of a compound may be practiced on any type of cell. In some embodiments, the cell may be a lymphocyte. Preferably, the cell is a mammalian cell. In some embodiments, the cell is a human peripheral blood lymphocyte. In
- 15 some embodiments, the cell is a murine cell, preferably a murine peripheral blood lymphocyte.
- The method of assessing clastogenicity of a compound may include preparing a chromosome spread. The method may include hybridizing one or more oligonucleotides to one or more chromosomes and enumerating chromosome spots. In some embodiments, one
- 20 or more of the oligonucleotides comprises a detectable moiety. Preferably, the detectable moiety is a fluorescent moiety although the detectable moiety may be one or more of biotin, digoxigenin, antigens, enzymes and haptens.
- The present invention also provides a method of assessing toxicity of a compound by contacting a cell with the compound, incubating the cell with a medium comprising a mitosis
- 25 enhancing factor, wherein the medium induces premature chromosome condensation and analyzing the condensed chromosomes. In some embodiments, the cell is contacted with the medium and the compound simultaneously. In other embodiments, the cell may be contacted with the compound and then transferred to a suitable medium. It may be desirable in some instances to incubate the cell after contact with the compound for a period of time sufficient to
- 30 allow chromosomal repair. In some embodiments, the mitosis enhancing factor may be one or more of cyclin kinases, histone kinases, cyclins, topoisomerases, SMC proteins, cdk1 substrate, histones, and components of mitosis promoting factor (MPF). In some preferred embodiments, the mitosis enhancing factor may include p34^{cdc2}/cyclin B kinase.
- A medium for use in the method of assessing toxicity of a compound may comprise a
- 35 phosphatase inhibitor. Preferably, the phosphatase inhibitor may be one or more of okadaic

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acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR.

5 The medium may comprise an energy source, preferably, ATP and/or GTP. The medium may include a transfection reagent.

The method of assessing toxicity of a compound may be practiced on any type of cell. In some embodiments, the cell may be a lymphocyte. Preferably, the cell is a mammalian cell. In some embodiments, the cell is a human peripheral blood lymphocyte. In some

10 The method of assessing toxicity of a compound may include preparing a chromosome spread. The method may include hybridizing one or more oligonucleotides to one or more chromosomes and enumerating chromosome spots. In some embodiments, one or more of the oligonucleotides comprises a detectable moiety. Preferably, the detectable moiety is a fluorescent moiety although the detectable moiety may be one or more of biotin, digoxigenin, antigens, enzymes and haptens.

15 The present invention also provides a method of detecting chromosomal abnormalities in a subject by isolating one or more cells from the subject, incubating the cell with a medium comprising a mitosis enhancing factor, wherein the medium induces premature chromosome condensation and analyzing the condensed chromosomes for abnormalities. In some embodiments, the mitosis enhancing factor may be one or more of cyclin kinases, histone kinases, cyclins, topoisomerases, SMC proteins, cdk1 substrate, histones, and components of mitosis promoting factor (MPF). In some preferred

20 embeddings, the mitosis enhancing factor may include p34^{cdc2}/cyclin B kinase. A medium for use in the method of detecting chromosomal abnormalities in a subject may comprise a phosphatase inhibitor. Preferably, the phosphatase inhibitor may be one or more of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR. The medium may comprise an energy source, preferably, ATP and/or GTP.

30 The medium may include a transfection reagent.

The method of detecting chromosomal abnormalities in a subject may be practiced on any type of cell. In some embodiments, the cell may be a lymphocyte. Preferably, the cell is a mammalian cell. In some embodiments, the cell is a human peripheral blood lymphocyte. In some embodiments, the cell is a murine cell, preferably a murine peripheral blood

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lymphocyte. In some embodiments, the cell may be obtained from a subject while the subject is *in utero*.

The method of detecting chromosomal abnormalities in a subject may include preparing a chromosome spread. The method may include hybridizing one or more oligonucleotides to one or more chromosomes and enumerating chromosome spots. In some embodiments, one or more of the oligonucleotides comprises a detectable moiety. Preferably, the detectable moiety is a fluorescent moiety although the detectable moiety may be one or more of biotin, digoxigenin, antigens, enzymes and haptens.

The present invention also provides a method of assessing a radiation dose received by a subject by isolating one or more cells from the subject, contacting one or more cells with a medium comprising a mitosis enhancing factor, wherein the medium induces premature chromosome condensation and analyzing the condensed chromosomes for abnormalities such as breakage, structural and/or numerical aberrations. In some embodiments, the mitosis enhancing factor may be one or more of cyclin kinases, histone kinases, cyclins, topoisomerases, SMC proteins, cdk1 substrate, histones, and components of mitosis promoting factor (MPF). In some preferred embodiments, the mitosis enhancing factor may include p34^{cdc2}/cyclin B kinase.

A medium for use in the method of assessing a radiation dose received by a subject may comprise a phosphatase inhibitor. Preferably, the phosphatase inhibitor may be one or more of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR. The medium may comprise an energy source, preferably, ATP and/or GTP. The medium may include a transfection reagent.

The method of assessing a radiation dose received by a subject may be practiced on any type of cell. In some embodiments, the cell may be a lymphocyte. Preferably, the cell is a mammalian cell. In some embodiments, the cell is a human peripheral blood lymphocyte. In some embodiments, the cell is a murine cell, preferably a murine peripheral blood lymphocyte.

The method of assessing a radiation dose received by a subject may include preparing a chromosome spread. The method may include hybridizing one or more oligonucleotides to one or more chromosomes and enumerating chromosome spots. In some embodiments, one or more of the oligonucleotides comprises a detectable moiety. Preferably, the detectable moiety is a fluorescent moiety although the detectable moiety may be one or more of biotin, digoxigenin, antigens, enzymes and haptens.

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The present invention also provides a composition comprising a cell and a cell culture medium, wherein the cell culture medium comprises a mitosis enhancing factor and induces premature chromosome condensation in the cell. In the compositions of the present invention, the mitosis enhancing may be one or more of cyclin kinases, histone kinases, 5 cyclins, topoisomerases, structural maintenance of chromosome (SMC) proteins, histones, *cdk1* substrate, and components of mitosis promoting factor. In some preferred embodiments, the mitosis enhancing factor may be p34^{cdc2}/cyclin B kinase. The compositions of the present invention may include a phosphatase inhibitor. The phosphatase inhibitor may be one or more of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, 10 cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR. The compositions may also comprise an energy source, preferably ATP and/or GTP.

The present invention provides kits for the induction of premature chromosome 15 condensation in test cells. In some embodiments, the kits may comprise one or more containers of a cell culture medium which comprises a mitosis enhancing factor and induces premature chromosome condensation in the cell. The mitosis enhancing may be one or more of cyclin kinases, histone kinases, cyclins, topoisomerases, structural maintenance of chromosome (SMC) proteins, histones, *cdk1* substrate, and components of mitosis promoting 20 factor. In some preferred embodiments, the mitosis enhancing factor may be p34^{cdc2}/cyclin B kinase. The kits of the present invention may include one or more containers holding one or more phosphatase inhibitors. The phosphatase inhibitor may be one or more of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin- 25 LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR. The kits may also comprise one or more containers holding an energy source, preferably ATP and/or GTP. The kits of the present invention may comprise one or more containers holding one or more transfection reagents.

30 **Brief Description of the Drawings**

Figure 1 is a schematic representation of the assembly and phosphorylation state of various mitosis enhancing factors in various stages of the cell cycle.

Figures 2A-2D show chromosome spreads of cells treated to induce premature chromosome condensation. Figure 2A is a photomicrograph of a Giemsa stained 35 chromosome spread of HPBLs in which premature chromosome condensation was induced by

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- mitogen stimulation and incubation in the presence of OA. Figure 2B is a photomicrograph of a Giemsa stained chromosome spread of HPBLs in which premature chromosome condensation was induced by incubation in the presence of p34^{cdc2}/cyclin B kinase and OA. Figure 2C is a photomicrograph showing a FISH analysis of chromosome 1 in un-irradiated
- 5 HPBLs in which premature chromosome condensation was induced by incubation in the presence of p34^{cdc2}/cyclin B kinase and OA. Figure 2D is a photomicrograph showing a FISH analysis of chromosome 1 in irradiated HPBLs in which premature chromosome condensation was induced by incubation in the presence of p34^{cdc2}/cyclin B kinase and OA.
- 10 Figure 3 is a graph showing the effects of various incubation times and OA concentrations on PCC index in mitogen stimulated HPBLs.
- Figure 4 is a graph showing the effects of various p34^{cdc2}/cyclin B kinase concentrations on PCC index in p34^{cdc2}/cyclin B kinase treated HPBLs.
- Figure 5 is a graph showing the dose-response curve for cells with radiation induced chromosome aberrations.
- 15 Figure 6 is a graph showing the increase in the percentage of cells with two or more fluorescent spots in cells isolated from patients exposed to radiation when compared to normal control cells.

Detailed Description of the Invention

- 20 The present invention provides materials and methods for the induction of premature chromosome condensation in without the need to stimulate the cells with a mitogen. In addition, the present invention provides methods of analyzing genetic material by inducing premature chromosome condensation and analyzing the physical structure of the condensed chromosomes. The present invention is useful in any application requiring premature
- 25 chromosome condensation in a test cell. The invention is particularly useful in the fields of cytogenetics, molecular cytogenetics, cell biology, genetic toxicology and genomics.
- In some aspects, the present invention provides materials and methods useful in diagnostic cytogenetics. The materials and methods of the present invention may be used in prenatal, postnatal and pre-implantation testing to evaluate the genetic material of a test cell.
- 30 For example, the methods described herein may be used to evaluate the genetic material in a potential sperm donor to determine the presence or absence of chromosomal aberrations in the sperm. Likewise, the present invention may be used to analyze the genetic material of a subject while the subject is *in utero*.
- In some related aspects, the present invention can be used in cytogenetic research. In
- 35 the field of genomics, for example, the present invention may be used to detect genes

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associated with various syndromes characterized by chromosomal aberrations, for example
Downs syndrome. In a particular embodiment, the present invention may be used to detect
genes associated with microdeletion syndromes. In another embodiment, the present
invention may be used to detect chromosomal anomalies (both numerical and structural)
5 associated with cancer. In some preferred embodiments, the present invention may be used to
detect gene amplification.

In the field of environmental testing, the present invention may be used to assess the
exposure of a subject to environmental insults. In some preferred embodiments, the present
invention may be used to assess the radiation dose received by a subject. The radiation dose
10 may have been received as a result of accidental exposure or may be the result of occupational
exposure. The present invention may be particularly useful in cases of exposure of a large
number of subjects as the capability of automating the present invention makes it well suited
to a high throughput automated screening system. In other embodiments, the exposure of a
subject to a compound which induces chromosomal abnormalities can be assessed.

15 In some preferred embodiments, the present invention provides methods of assessing
the toxicity of a drug. These methods are useful in the identification of potential
chemotherapeutic agents where it is desirable to have an agent capable of inducing
chromosomal breaks. In this aspect, the present methods may be used to assess the
clastogenicity (ability to break chromosomes) of a particular agent. The present methods may
20 also be used as an initial safety screen to determine whether a therapeutic agent induces
chromosomal aberrations.

Cells

Any type of cell having genetic material may be used in the practice of the present
25 invention. For example, cells from heart, lung, liver, kidney, brain or other tissue may be
used as a source of cells. The isolation of cells from various tissues may be accomplished
using any technique known to those skilled in the art. In preferred embodiments, the cells are
of mammalian origin, such as human or murine cells. In some preferred embodiments,
peripheral blood lymphocytes may be used for premature chromosome condensation and
30 analysis. In other preferred embodiments, cells may be oocytes or obtained from embryos,
amniotic fluid or established cell lines, such as stem cell lines.

The isolation of the cells to be used in the present invention may be by any means
known to those skilled in the art. In some preferred embodiments, human peripheral blood
lymphocytes (HPBLs) may be used. The isolation of peripheral blood lymphocytes is routine
35 in the art. One suitable protocol is described below and other methods known to those skilled

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- in the art could be used. In the following protocol, the peripheral blood lymphocytes were isolated from a human subject. They could equivalently be isolated from any subject. In some preferred embodiments, the subject may be mammalian. In other preferred embodiments, the subject may be a human or a mouse.
- 5 Lymphocytes may be isolated from whole blood samples using any suitable technique known to those skilled in the art. An example of a suitable technique is density gradient centrifugation, for example, using Histopaque 1077 (Sigma Chemical Co.). After centrifugation, cells may be collected and washed twice in phosphate-buffered saline (pH 7.0). The cells may then be re-suspended in a suitable cell culture medium. The selection of
- 10 a suitable cell culture medium for a given type of cell is routine in the art. When the cells are lymphocytes, a suitable medium may be Karyomax (Life Technologies Inc.). The cells may be re-suspended at a concentration suitable for subsequent analysis, for example, at a concentration of from about 1×10^6 cells per ml to about 1.5×10^6 cells per ml before use.
- 15 Cell Culture Media
- The present invention provides a cell culture medium for inducing premature chromosome condensation in a test cell. Any suitable cell culture medium may be supplemented with one or more mitosis enhancing factors to be used as a cell culture medium of the invention. A suitable cell culture medium is one in which the cell of interest may be
- 20 maintained in a viable state throughout the duration the induction of premature chromosome condensation. Optionally, the suitable cell culture medium may be one in which the test cell may be maintained for a protracted period of time.
- The cell culture media of the present invention will typically comprise various ingredients selected to maintain the viability of the test cells. Such ingredients include, but
- 25 are not limited to, amino acids, vitamins, inorganic salts, buffers or buffer salts, sugars, lipids, trace elements, cytokines and hormones. Suitable cell culture media are commercially available from, for example, Life Technologies Inc.
- In preferred embodiments, a cell culture medium of the present invention will comprise one or more mitosis enhancing factors. Mitosis enhancing factors are agents
- 30 associated with the progression of the cell cycle into mitosis. Mitosis enhancing factors include, but are not limited to, cyclins, cyclin kinases, histone kinases, topoisomerases, SMC proteins, cdk1 substrate, histones, and components of mitosis promoting factor (MPF). In preferred embodiments, the mitosis enhancing factor may be a purified mitosis enhancing factor. The mitosis enhancing factor may be purified to any desired level of purity.
- 35 Preferably, the mitosis enhancing factor will at least 50% pure, *i.e.*, the mitosis enhancing

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factor will make up at least 50% by weight of a mitosis-enhancing-factor-containing material to be added to a culture medium. In other preferred embodiments, a mitosis enhancing factor may be 75% or greater pure, 80% or greater pure, 85% or greater pure, 90% or greater pure or 95% or greater pure. In a preferred embodiment, a cell culture medium of the present invention may comprise p34^{cdc2}/cyclin B kinase. Suitable p34^{cdc2}/cyclin B kinase is commercially available from, for example, New England Biolabs.

The mitosis enhancing factor may be added to the medium alone or in combination with other factors. The mitosis enhancing factor may be in the form of a native protein or a mutagenized protein. For example, fusion proteins comprising a mitosis enhancing factor may be used. A mitosis enhancing factor may be placed in frame with a protein or peptide portion of a different protein to produce a fusion protein. The construction of fusion proteins is routine in the art (see, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press). In preferred embodiments, the fusion proteins of the present invention may comprise, in addition to a mitosis enhancing factor, one or more ligands for a receptor to facilitate cellular uptake of the fusion protein, nuclear localization signals, purification tags, epitopes or the like. In a preferred embodiment, a cell culture medium of the present invention may comprise a fusion protein comprising a mitosis enhancing factor and a nuclear localization sequence. Suitable nuclear localization signals are known in the art and may be found, for example, in United States Patents 6,051,429 and 5,736,392.

In addition to mitosis enhancing factors, a cell culture medium of the present invention may comprise one or more energy sources including, but not limited to, ATP and GTP.

A cell culture medium of the present invention may optionally comprise one or more transfection reagents. As used herein, transfection reagent is seen to include any reagent which, when added to a cell culture medium, enhances the uptake by a test cell of a mitosis enhancing factor. Transfection reagents include, but are not limited to, neutral lipids, cationic lipids, mixtures of neutral and cationic lipids, proteins, peptides, lipoproteins, lipopeptides and the like. Suitable transfection reagents may be obtained commercially from, for example, Promega Inc. and Life Technologies Inc. In some preferred embodiments, the transfection reagents of the present invention may comprise a peptide that enhances receptor mediated endocytosis. Examples of such transfection reagents may be found in United States Patent 6,103,529. The transfection reagent may be added directly to the media or may be combined with the mitosis enhancing factor prior to the addition of the mitosis enhancing factor to the medium.

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A cell culture medium of the present invention may optionally comprise one or more phosphatase inhibitors. In some preferred embodiments, the protein phosphatases may specifically inhibit serine/threonine protein phosphatases. In some preferred embodiments the phosphatase inhibitors may specifically inhibit the protein phosphatases 1 and 2A. Suitable protein phosphatase inhibitors include, but are not limited to, okadaic acid, salts of okadaic acid, 5 calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR.

10 Cell Culture Compositions

The cell culture media of the present invention may be used to formulate cell culture compositions comprising a cell or cell population and a culture medium of the invention. The cell may be any cell in which it is desired to induced premature chromosome condensation. Cells isolated from subjects are particularly preferred. The isolated cells may be derived from 15 any organ or tissue in the subject including, but not limited to, blood, heart, lung, epithelial tissue and/or intestinal tissue.

Kits

The present invention contemplates kits adapted for use in cytogenetic research. 20 Typically, the kits of the invention may comprise one or more containers holding a cell culture medium of the present invention. The cell culture medium may be in liquid form or in the form of a dry powder concentrate. The kits of the invention may comprise one or more containers holding one or more mitosis enhancing factors. The factors may be in solution or may be in the form of a dried powder. Kits of the invention may comprise one or more 25 containers holding one or more phosphatase inhibitors. Optionally, kits of the invention may comprise one or more containers holding one or more transfection reagents and/or one or more energy sources which may be in solution or in dry form.

Kits of the present invention preferably comprise instructions for inducing premature chromosome condensation using the materials and methods of the present invention. In 30 particular, the instructions may provide detailed protocols for inducing premature chromosome condensation in a cell or cell population without the need to stimulate the cell or cell population with a mitogen.

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Preparation and analysis of chromosome spreads

PCC spreads may be prepared according to standard cytogenetic procedures directly after the indicated treatment. Briefly, cells may be treated with a hypotonic potassium chloride (0.075 M) solution for 5 minutes and fixed in acetic: methanol (1:3) fixative. Fixed cells may be dropped onto acid cleaned glass slides.

To directly visualize the spread chromosomes, the slides may be stained. Suitable stains are known to those of skill in the art, for example, a 4% aqueous solution of Giemsa stain may be used for observation under a light microscope. Coded slides can be analyzed under 1000 × magnification. Cells with condensed chromatin material displaying at least partial separation of chromosomes are scored as PCC spreads.

The PCC index may be determined as follows.

$$\frac{\text{PCC spreads number}}{(\text{interphase cell number} + \text{PCC spreads number})} \times 100$$

For experiments involving fluorescent *in situ* hybridization analysis (FISH), after preparing a chromosome spread, whole-chromosome DNA hybridization probe specific for one or more chromosomes. Optionally, a whole chromosome DNA hybridization probe may be directly labeled with a detectable moiety and may be used to analyze the spread chromosomes. Such labeled chromosome-probes are commercially available. As an example, whole chromosome probe specific for chromosome 1 labeled with spectrum green fluorochrome may be obtained from Vysis Inc.

In situ hybridization and chromosome painting may be done using techniques well known in the art (see, for example, Brown *et al.* (1992) *Int. J. Radiat. Oncol. Biol. Phys.* 24, 279-286).

In the working example of the invention disclosed below, a chromosome 1 probe from Vysis was used according to the manufacturer's protocol. Other suitable probes are known to those skilled in the art and may be used without departing from the spirit of the invention. Other preferred probes include probes specific for pathological conditions.

Cells may be mounted in a medium containing 4,6-diamidino-2-phenyl-indole (DAPI) for analyzing chromosome 1 aberrations under a fluorescence microscope (Leitz) equipped with filters for DAPI and fluorescein isothiocyanate (FITC).

The coded slides may be observed at a 1000 × magnification for analyzing aberrations involving chromosome 1. Chromosome aberration analysis is based on the following general criteria:

The cells to be included in the analysis should show one or more (and preferably all) of the following: (a) at least partial separation of chromosomes with condensed chromatin

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material as determined by DAPI counterstain, (b) two or more clearly separated chromosome 1-specific spots with bright green fluorescent signals (cells with single green spots, arising because of overlapping signals, were not included), (c) spots that were similar in fluorescent intensity, and (d) an area representing about 15 to 100% of the area of the spots observed in samples from sham-treated controls.

The area of spots in the control samples may not always be uniform because of differential chromosome condensation and, in a few cases, angular presentation under the microscope. In such cases of ambiguity, cells should be excluded from analysis.

It will be readily apparent to those of skill in the art that other suitable modifications and adaptations may be made to the materials and methods of the present invention without departing from the scope of the invention or any embodiment thereof. Having now described the invention in detail, the invention may be more clearly understood with reference to the figures and the following non-limiting examples.

15 Examples

Example 1

Induction of premature chromosome condensation in mitogen stimulated cells

For purposes of comparison and in order to determine a suitable level of phosphatase inhibitor, premature chromosome condensation was induced in HPBLs using prior art methodology.

HPBLs prepared as described above may be incubated in cell culture medium supplemented with an energy source. In order to determine the optimal OA concentration and duration of incubation for PCC, phytohemagglutinin (PHA, 10 $\mu\text{g}/\text{ml}$; Murex Diagnostics) was subsequently added to the medium to stimulate proliferation. This complete medium did not contain a mitosis enhancing factor.

Incubation of unstimulated HPBL in a cell culture medium containing OA alone did not result in PCC induction, thus, PHA was used to help activate cell cycle progression. The HPBL were treated with OA at concentrations ranging from 0.25 to 1 μM in a cell culture medium containing 100 μM ATP and incubated at 37°C for varying durations of up to 24 hours. Slides were prepared and PCC index was determined as explained above.

Figure 2A is a representative photomicrograph showing PCC induced by a treatment with OA in a mitogen-stimulated HPBL stained with Giemsa. Dissolution of cell membrane, condensation of the chromatin material, and partial separation of chromosomes characterized OA-induced PCC. Undivided chromosomes appear less condensed compared to metaphase

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chromosomes or PCC induced by mitotic-cell fusion technique, and chromosome clumps are still visible in most cells.

Figure 3 shows the effect of OA concentration and duration of incubation on PCC induction in the mitogen-stimulated HPBL model. Pooled data is shown from two or more independent experiments with each concentration and time point representing more than 1,000 cells. Treatment of mitogen-stimulated HPBL with OA (0.25 μ M) resulted in significant ($p < 0.01$, Student's t-test) PCC levels determined by PCC index within 1 hour, compared with controls. The PCC index reached a maximum of 61% at a 1 μ M concentration at 8 hours. At a 0.75- μ M concentration, the index peaked at 2 hours, exhibiting PCC in about 20% of cells, and remained at that level for up to 24 hours. It appears that OA at 0.75 μ M concentration is not cytostatic and induces a reasonably high PCC yield in mitogen-stimulated HPBL model. Therefore, this concentration was used in further studies with p34^{cdc2}/cyclin B kinase to induce PCC in unstimulated HPBL.

It has been previously demonstrated that treatment of mitogen-stimulated HPBL with phosphatase inhibitors, such as OA or calyculin A, induces premature condensation of chromatin material. In those studies, HPBL were treated, 41 to 45 hours after PHA stimulation, with OA doses between 0.1 and 0.5 μ M (Gotoh *et al.* (1996) *Int. J. Radiat. Biol.* 70, 517-520; Kanda *et al.* (1999) *Int. J. Radiat. Biol.* 75, 441-446) or with 0.05 μ M calyculin A (Durante *et al.* (1998) *Int. J. Radiat. Biol.* 74, 457-462) for varying durations of 1 to 6 hours to induce entry into a mitosis-like state from the S- or G₂-phase of the cell cycle. In the present experiment, the effects of OA concentrations between 0.25 and 1 μ M, treated immediately and up to 24 hours after mitogen stimulation of HPBL were studied. PHA was used to help activate cell cycle progression. In this study, significant ($p < 0.01$) elevation in PCC yield was observed as early as 1 hour, indicating PCC induction before DNA replication in a rapidly dedifferentiating cohort of mitogen-stimulated HPBL population. The significant ($p < 0.01$) elevation in PCC index that was observed as early as 1 hour after treatment with OA is comparable to that seen in proliferating cells by others (Gotoh *et al.* (1996) *Int. J. Radiat. Biol.* 70, 517-520; Durante *et al.* (1998) *Int. J. Radiat. Biol.* 74, 457-462; Coco-Martin *et al.* (1997) *Int. J. Radiat. Biol.* 71, 265-273; and Ghosh *et al.* (1992) *Exp. Cell Res.* 201, 535-540).

In the optimization study (Figure 3), 0.75 μ M OA resulted in a peak PCC level of 20% at 2 hours and remained at that level for up to 24 hours. This dose was used for treatment with p34^{cdc2}/cyclin B kinase to induce PCC in the unstimulated HPBL model. Selection of this dose was based not only on PCC yield but also on quality of PCC spreads. Similar to the observations of Kanda *et al.* (Kanda *et al.* (1999) *Int. J. Radiat. Biol.* 75, 441-

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446), prolonged treatment with higher concentrations of OA was observed to result in poor spread quality, possibly due to toxicity. In addition, OA was found to arrest cell cycle progression in human myeloid leukemic cell lines in a concentration- and time-dependent manner (Ishida *et al.* (1992) *J. Cell. Physiol.* 150, 484-492). At higher PCC inducible concentrations (above 0.5 μM), cell-cycle arrest occurred at G₁-S-phase; but in lower concentrations cell-cycle arrest occurred at G₂-M phase (Ishida *et al.* (1992) *J. Cell. Physiol.* 150, 484-492).

Example 2

10 Induction of premature chromosome condensation in resting cells

In the following working example of the present invention, PCC induction in unstimulated HPBL was accomplished by the addition of p34^{cdc2}/cyclin B kinase to the complete media supplemented with ATP (100 μM) containing OA (0.75 μM) and incubation for three hours at 37°C. PCC index was determined from two or more independent experiments, each data point representing more than 1,000 cells. The pooled data were compared with the yield obtained by OA treatment alone in the mitogen-stimulated HPBL model. The results obtained were compared to the results obtained using the prior art methodology of the preceding example.

The presence of p34^{cdc2}/cyclin B kinase at concentrations as low as 5 units per ml resulted in PCC induction in unstimulated HPBL. At this concentration, the PCC yield was approximately 30% higher than the yield in the group treated with OA alone in mitogen-stimulated HPBL (Figure 4). An increase in the enzyme concentration resulted in a concentration-dependent and significant ($p < 0.05$; Student's t-test) increase in PCC yield (Figure 4). It also improved the spreading and condensation of the chromatin material, yielding better quality PCC spreads (Figure 2B).

Example 3

30 Determination of radiation dosage using chromosome spreads from unstimulated cells

The PCC spreads prepared from unstimulated cells were suitable for detecting radiation-induced chromosome aberrations involving a specific chromosome after hybridization with whole-chromosome probes by the "spot assay" described by Coco-Martin and Begg (Coco-Martin *et al.* (1997) *Int. J. Radiat. Biol.* 71, 265-273).

Cell suspension in Karyomax was placed in 15-ml polypropylene centrifuge tubes and, at room temperature, was exposed to gamma rays at a dose rate of 1 Gy/min in a bilateral field of a ⁶⁰Co facility. Radiation source and dosimetry procedures were previously described

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(Stankus *et al.* (1995) *Int. J. Radiat. Biol.* 68, 1-9). The dose rate was measured with a tissue-equivalent ionization chamber before irradiation. The field was uniform within 2%. In radiation dose-response studies, unstimulated HPBL were incubated at 37°C for 21 hours after exposure in complete medium before PCC induction.

5 FISH was used to quantify cells with radiation-induced structural aberrations involving chromosome 1 in PCC spreads obtained by incubating unstimulated HPBL in a medium containing OA, ATP, and p34^{cdc2}/cyclin B kinase. The study evaluated the potential application of this "spot assay" to biological dosimetry and included a 24 hour repair
10 incubation at 37°C following exposure to gamma-ray doses of 0 to 7.5 Gy. PCC spreads were prepared and FISH technique was applied as explained above. Since the maximum difference between experiments was not significant (chi-square value = 0.265, p = 0.606 for one degree
15 freedom), the data were pooled from four independent experiments, with each dose level representing two or more experiments. At least 1,000 cells were analyzed for enumerating aberrations involving chromosome 1.

15 In cells that had not been irradiated, two fluorescent (green) spots were seen, reflecting two copies of chromosome 1 (Figure 2C). Irradiated cells often exhibited more than two fluorescent spots (Figure 2D) due to induction of aberrations in chromosome 1, which likely reflect radiation-induced fragments or exchanges. The data on frequency
20 distribution of cells with aberrations involving chromosome 1, after exposure to different doses of gamma radiation, are presented in Table 1.

20 These data demonstrate that the number of cells with aberrant chromosome 1 increases with radiation doses between 0 and 7.5 Gy. This, in general, is in good agreement with dose-effect increase for cytogenetic endpoints. The number of chromosome 1 excess spots increased with radiation dose from 0.035 ± 0.0058 per cell at 0.5 Gy to 0.236 ± 0.0126
25 at 7.5 Gy. Base-line frequency of cells with chromosome 1 aberrations in FISH-painted PCC spreads was 0.006 ± 0.0020. Frequency of cells with two spots decreased from 0.965 at 0.5 Gy to 0.803 at 7.5 Gy with a corresponding increase in the frequency of cells with more than two spots (Table). The number of cells with more than two spots for chromosome 1 increased
30 with radiation dose from 0 to 7.5 Gy and reached a maximum of 19.70 ± 1.258 per cent (Figure 5).

The dose-response data for the number of cells with aberrant chromosome 1 were fitted with two models, a linear model ($Y = (2.77 \pm 0.230) D + 0.90 \pm 0.431$ and $r^2 = 0.966$) fitted by the weighted least-squares regression method (weights were based on the reciprocal of the SE of the mean squared) and a nonlinear power model ($Y = (5.70 \pm 0.46) D^{(0.61 \pm 0.05)}$)

Table 1
Frequency distribution analysis of cells with aberrant chromosome 1 after exposure to different doses of gamma rays as visualized in PCC induced by a treatment with okadaic acid and p34^{cdc2} cyclin B kinase^a

Radiation dose (Gy)	Total number of cells	Frequency of cells with number of chromosome 1 spots				Frequency of cells with > 2 spots Mean ± SE	Number of excess spots/cell Mean ± SE
		2 spots	3 spots	4 spots	5 spots		
0	1500	0.994	0.006	—	0.006 ± 0.0020	0.006 ± 0.0020	
0.5	1000	0.965	0.035	—	0.035 ± 0.0058	0.035 ± 0.0058	
1.5	1000	0.917	0.083	—	0.083 ± 0.0087	0.083 ± 0.0087	
3.0	1003	0.890	0.1096	—	0.110 ± 0.0099	0.110 ± 0.0099	
4.5	1486	0.869	0.1232	0.008	0.131 ± 0.8760	0.139 ± 0.0088	
6.0	1666	0.828	0.1477	0.0240	0.172 ± 0.0092	0.196 ± 0.0092	
7.5	1000	0.803	0.1580	0.0390	0.197 ± 0.0126	0.236 ± 0.0126	

^aData were pooled from four independent experiments with each dose level representing two or more experiments.

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and $r^2 = 0.9901$). When fitted with a nonlinear power model, a bending of the dose-response curve towards the abscissa was observed.

The dose-response relationship has a broader dose range than other metaphase-spread based cytogenetic assays or micronucleus assay. With the nonlinear power model fit, the observed downward curvature of the dose-response curve towards the abscissa. Since only one chromosome pair was painted, which represents only a fraction of the genome, some saturation of the signal was expected with increasing radiation dose. This effect is particularly true at higher radiation doses where the number of separate signals produced by complexes (both exchanges and fragments) is restricted, with nuclear area being constant for a given cell. In addition, mean exchanges per cell are known to increase with a positive upward curvature with low-LET radiation. In this case, this curvature was somewhat mitigated because of the inclusion of fragments (which have different dose-response curves) that distorted the curve. The better fit with a nonlinear power model suggests that this assay may be more sensitive at lower radiation doses. This data is in good agreement with earlier data of Coco-Martin and Begg (Coco-Martin *et al.* (1997) *Int. J. Radiat. Biol.* 71, 265-273), which involved a measurement of chromosome 4 aberrations induced by gamma irradiation in a human adenocarcinoma cell line (A549) in G₁-phase PCC induced by OA.

Example 4

20 In vivo validation of determination of radiation dosage

The methods disclosed herein can be used to assess the dose of radiation received by a subject. This was demonstrated using premature chromosome condensation spreads of HPBLs performed after a 24 hour repair incubation at 37°C following exposure to different doses of gamma rays. A base-line frequency of 0.006 ± 0.0020 per cell involving chromosome 1 aberrations was observed in unstimulated HPBL for this assay. This is higher than base-line frequencies for other cytogenetic assays (e.g., dicentric (0.001 per cell) measured in metaphase spreads). A higher base-line frequency, in general, suggests that some cells carrying aberrations are lost from the cell population before mitosis and, therefore, are not detected by the metaphase-spread-based cytogenetic assays. Thus, the present methods more accurately assess the condition of the cells, since cells that are not competent to undergo mitosis are still represented in the data set and are not lost.

HPBL samples were collected from individuals who had been exposed to ⁶⁰Co gamma radiation from a scrap metal source, a radiation leak occurring in Bangkok, Thailand. These individuals received radiation doses of 0.1 to 16 Gy, at a dosage rate of up to 200 μSv/h. From those exposed to the radiation (over 30 people), twelve samples were collected

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approximately four months after exposure, and nine samples with controls were analyzed by the FISH method described above to determine the number of chromosomal aberrations in chromosome 1. These data are presented in Figure 6, which shows the increase in the percentage of cells with two or more fluorescent spots in cells isolated from patients exposed to radiation when compared to normal control cells.

5 The methods of the present invention, as exemplified by the PCC assay performed on HPBL of exposed individuals, provide a direct and sensitive cytogenetic tool for biodosimetry (Pantelias *et al.* (1985) *Mutat. Res.* 149, 67-72; Prasanna *et al.* (1997) *Health Phys.* 72, 594-600; and Cornforth *et al.* (1983) *Science* 222, 1141-1143). The assay can rapidly predict
10 absorbed dose (within 24 hours of the receipt of a blood sample in the laboratory) to enable effective clinical treatment. Since it is conducted on unstimulated cells and does not require cell division, confounding factors such as radiation-induced cell-cycle delay (Poncelet *et al.* (1988) *Strehlanther. und Onkol.* 164, 542-543) and death (MacVittie *et al.* (1996) *Advances in the Treatment of Radiation Injury*, Elsevier Science, 263-269) do not interfere with dose
15 estimates.

These results indicate that the present method provides a simpler and more reliable techniques for biological dosimetry of radiation exposures than currently used techniques such as analysis of chromosome aberrations in metaphase or PCC spreads after mitotic-cell fusion. The present method involves inducing PCC in unstimulated cells and analyzing
20 aberrations involving specific chromosomes. This method, involving a simple incubation of test cells in a cell culture medium containing a mitosis enhancing factor and optionally a phosphatase inhibitor and an energy source (for example, p34^{cdc2}/cyclin B kinase, OA and ATP), to induce premature chromosome condensation, is simple and does not require the high degree of technical expertise associated with alternative PCC-inducing protocols (Pantelias *et al.* (1983) *Somatic Cell Genet.* 9, 533-547; Johnson *et al.* (1970) *Nature* 226, 717-722).
25

Example 5

Examination of chromosomal integrity in oocytes, blastocysts, stem cells and embryonic cells

Using the methods in Example 2, PCC is induced in a single cell, such as an oocyte,
30 polar body or cell from a blastocyst, or multiple cells, such as an amniotic fluid sample or cells from an established human stem cell line. Oocytes or embryonic cells from mice can also be used. The cell or cells are incubated in the complete medium described in Example 2 for 3 hours at 37°C. Chromosome spreads are prepared, and the chromosomes are examined using any of the methods described on page 15. Structural abnormalities are indicated, *e.g.*,
35 by more than 2 bright fluorescent spots, using the FISH technique, or by failure of a locus

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specific probe to bind to a chromosome. Healthy embryos or cell lines are maintained in culture or *in utero*, and healthy oocytes, whose corresponding polar bodies are tested, are fertilized. Abnormal cells are not maintained in culture or used in further procedures.

- For optimization of the complete medium, samples containing multiple cells can be split into portions, each of which is incubated in the complete medium of Example 2, but in which each portion contains a different concentration of a phosphatase inhibitor (okadaic acid or calyculin A) or an energy source (ATP) or a cyclin kinase (p34^{cdc2}/cyclin B kinase). Multiple components can be optimized, according to the number of sample portions available. After three hours at 37°C, the cells are harvested, subjected to hypotonic treatment, fixed with methanol/acetic acid, placed on slides and stained to obtain chromosome spreads. The percentage of cells in which PCC is induced is calculated for each sample, and a dose-response relationship is determined. The optimal concentration of one or more components is then used to prepare complete medium for subsequent analyses.

- Micromanipulation techniques are used to manipulate single-cell embryos or oocytes. The cell is held attached to a micropipette tip and contained in a culture dish with complete medium. The cell is incubated in medium for several hours at 37°C prior to induction of PCC. Alternatively, a solution of p34^{cdc2}/cyclin B kinase and either okadaic acid or calyculin A is introduced into the cell by microinjection or by electroporation. The contents of the dish are then replaced with, successively, hypotonic solution and fixative, and a chromosome spread is prepared. As a second alternative, the cell is held within a capillary tube containing complete medium for incubation, and the aforementioned treatments performed by aspiration and refilling. This procedure is carried out under a stereomicroscope. A chromosome spread is prepared in a similar fashion.

- The chromosomes are examined by *in situ* hybridization, chromosome painting or fluorescence microscopy, as described above. Whole-chromosome DNA hybridization, in which the chromosome is labeled with a commercially available fluorochrome, is specific for single chromosomes. *In situ* hybridization and chromosome painting are carried out according to standard methods. Following PCC induction, the cell sample is mounted in medium containing DAPI under a fluorescence microscope equipped with filters for DAPI and FITC. Chromosome aberrations, such as those studied in chromosome 1, are visible and can be analyzed for type and number.

Example 6

High-throughput isolation of PCC-sensitive lymphocyte subpopulations

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For cytogenetic applications and analyses involving large numbers of samples, high-throughput procedures for isolating subpopulations of lymphocytes that are susceptible to PCC are required. Currently procedures are tedious and inefficient, *i.e.*, isolation on a density gradient (*e.g.*, Ficoll, Histopaque), followed by treatment with a mitogen and PHA.

5 Metaphase spreads are then prepared, and the cycle arrested by treatment with colcemid. These cells are then cultured, all to produce a subpopulation with a mitotic yield of 4-5%.

To quickly and simply produce adequate numbers of PCC-sensitive peripheral blood lymphocytes, whole blood is mixed with a cocktail containing RosetteSep[®] (Stem Cell Technologies) multivalent antibodies in centrifuge tubes, *e.g.*, 50 ml conical centrifuge tubes.

10 The tubes are incubated for 20 minutes at room temperature. Mitogen and PCC-insensitive lymphocytes and non-lymphocytic white blood cells are cross-linked by the antibodies to form tetrameric "rosette" complexes. The contents in each tube are then underlaid with Ficoll, and the tubes are spun for 20 minutes. An interface containing a purified lymphocyte subpopulation that is PCC-sensitive is formed between an upper plasma layer and a lower
15 Ficoll layer. Unwanted white blood cells, red cells and other cellular and particulate blood components are pelleted to the bottom.

This procedure is scalable to include a large number of blood samples (>500 per run using an automated isolation system), and a ten-fold increase mitotic yield is achievable. As a result, this procedure is preferable to current methods for cytogenetic applications. For
20 clinical applications related to immune system disorders, this procedure is well-suited for the isolation of T cell subpopulations such as CD3+ T cells, CD4+ T cells and CD8+ T cells.

Isolation of PCC-sensitive lymphocyte subpopulations is also accomplished using StemSep[®] (Stem Cell Technologies) immunomagnetic cell selection assay. In this assay, the reagent cocktails consist of antibodies directed against markers present on the surface of the
25 unwanted cells in the sample. The cells labeled by these antibodies are efficiently removed by passage through a magnetic column, while the desired cells are collected in the column flow through, unlabeled and highly enriched. StemSep[®] immunomagnetic negative cell selection is used for isolation of memory CD4+ T cells (CD4+ T cell cocktail plus CD45 RA),
30 Resting CD4+ T cell (CD4+ T cell cocktail plus one or more of CD25, CD69, HLA-DR), Resting CD8+ T cell (CD8+ T cell cocktail plus one or more of CD25, CD27, CD69, HLA-DR), $\alpha\beta$ T cell (T cell cocktail plus TCR $\gamma\delta$) and $\gamma\delta$ T cell (T cell cocktail plus TCR $\alpha\beta$).

Having fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide
35 and equivalent range of conditions, formulations and other parameters without affecting the

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scope of the invention or any specific embodiment thereof. Any such modifications or changes are intended to be within the scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains and are specifically incorporated herein by reference.

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What is claimed:

1. A culture medium for inducing premature chromosome condensation in a cell comprising a cyclin kinase having mitosis enhancing properties, wherein the cyclin kinase is present in an amount effective to induce premature chromosome condensation.
2. A medium according to claim 1, wherein the cyclin kinase is p34^{cdc2}/cyclin B kinase.
3. A medium according to claim 1, further comprising a phosphatase inhibitor.
4. A medium according to claim 3, wherein the phosphatase inhibitor is selected from a group consisting of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothal, fenvalerate, fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR.
5. A medium according to claim 1, further comprising an energy source.
6. A medium according to claim 5, wherein the energy source is selected from a group consisting of ATP and GTP.
7. A medium according to claim 1, further comprising a transfection reagent.
8. A kit comprising the medium of any one of claims 1-7.
9. A method of analyzing a chromosome, comprising:
 - (a) incubating a cell with a medium comprising a cyclin kinase having mitosis enhancing properties, wherein the cyclin kinase is present in an amount effective to induce premature chromosome condensation; and
 - (b) analyzing the condensed chromosome.
10. A method of assessing clastogenicity of a compound, comprising:
 - (a) contacting a cell with the compound;

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- (b) incubating the cell with a medium comprising a cyclin kinase having mitosis enhancing properties, wherein the cyclin kinase is present in an amount effective to induce premature chromosome condensation; and
- (c) analyzing the condensed chromosomes for breakage, structural and/or numerical aberrations.
- 5
11. A method according to claim 10, wherein the cell is contacted with the medium and the compound simultaneously.
- 10
12. A method according to claim 10, further comprising incubating the cell after contact with the compound for a period of time sufficient to allow chromosomal repair.
13. A method of assessing toxicity of a compound, comprising:
- (a) contacting a cell with the compound;
- 15 (b) incubating the cell with a medium comprising a cyclin kinase having mitosis enhancing properties, wherein the cyclin kinase is present in an amount effective to induce premature chromosome condensation; and
- (c) analyzing the condensed chromosomes.
- 20
14. A method according to claim 13, wherein the cell is contacted with the medium and the compound simultaneously.
- 15
15. A method according to claim 13, further comprising incubating the cell after contact with the compound for a period of time sufficient to allow chromosomal repair.
- 25
16. A method of detecting chromosomal abnormalities in a subject, comprising:
- (a) isolating one or more cells from the subject;
- (b) contacting at least one cell with a medium comprising a cyclin kinase having mitosis enhancing properties, wherein the cyclin kinase is present in an amount effective to
- 30 induce premature chromosome condensation; and
- (c) analyzing the condensed chromosomes for chromosome abnormalities.
17. A method according to claim 16, wherein chromosomal abnormalities are
- 35 analyzed based on an evaluation of the number of condensed chromosome domains or spots within a cell.

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18. A method according to claim 17, wherein the subject is *in utero*.
19. A method according to claim 17, wherein the abnormality is a numerical
5 abnormality.
20. A method according to claim 17, wherein the abnormality is a structural
abnormality.
- 10 21. A method of assessing a radiation dose received by a subject, comprising:
(a) isolating one or more cells from the subject;
(b) contacting at least one cell with a medium comprising a cyclin kinase having
mitosis enhancing properties, wherein the cyclin kinase is present in an amount effective to
induce premature chromosome condensation; and
15 (c) analyzing the condensed chromosomes for chromosome abnormalities.
22. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the cyclin
kinase is p34^{cdc2}/cyclin B kinase.
- 20 23. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the
medium further comprises a phosphatase inhibitor.
24. A method according to claim 23, wherein the phosphatase inhibitor is selected
from a group consisting of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid,
25 cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate,
fostriecin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-
RR, and microcystin-YR.
25. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the
30 medium further comprises an energy source.
26. A method according to claim 25, wherein the energy source is selected from a
group consisting of ATP and GTP.

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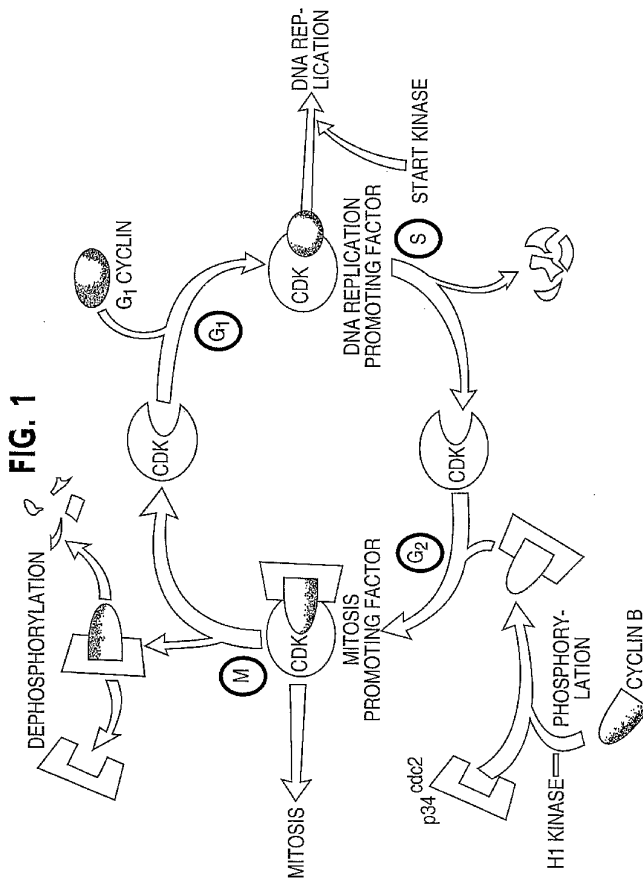
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27. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the medium further comprises a transfection reagent.
28. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the cell is a lymphocyte.
5
29. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the cell is a mammalian cell.
- 10 30. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the cell is a human peripheral blood lymphocyte.
31. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the cell is a murine cell.
15
32. A method according to any one of claims 9, 10, 13, 16 or 21, wherein the cell is a murine peripheral blood lymphocyte.
33. A method according to any one of claims 9, 10, 13, 16 or 21, wherein analyzing the chromosome comprises preparing a chromosome spread.
20
34. A method according to any one of claims 9, 10, 13, 16 or 21, wherein analyzing the chromosomes comprises hybridizing an oligonucleotide to at least one or more chromosomes and enumerating chromosome spots.
25
35. A method according to claim 34, wherein the oligonucleotide comprises a detectable moiety.
36. A method according to claim 35, wherein the detectable moiety is a fluorescent moiety.
30
37. A method according to claim 35, wherein the detectable moiety is selected from a group consisting of biotin, digoxigenin, antigens, enzymes and haptens.

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38. A composition comprising a cell and a cell culture medium comprising a cyclin kinase having mitosis enhancing properties, wherein the cyclin kinase is present in an amount effective to induce premature chromosome condensation.
- 5 39. A composition according to claim 38, wherein the cyclin kinase is p34^{cdc2}/cyclin B kinase.
40. A composition according to claim 38, further comprising a phosphatase inhibitor.
- 10 41. A composition according to claim 40, wherein the phosphatase inhibitor is selected from a group consisting of okadaic acid, salts of okadaic acid, calyculin A, cantharidic acid, cantharidin, cypermethrin, deltamethrin, dephostatin, 3,4-dephostatin, endothall, fenvalerate, fostriccin, microcystin-LA, microcystin-LF, microcystin-LR, microcystin-LW, microcystin-RR, and microcystin-YR.
- 15 42. A composition according to claim 38, further comprising an energy source.
43. A composition according to claim 42, wherein the energy source is selected from a group consisting of ATP and GTP.
- 20 44. A composition according to claim 38, further comprising a transfection reagent.
45. A kit comprising the composition of any one of claims 38-44.



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FIG. 2B



FIG. 2D



FIG. 2A

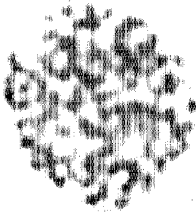
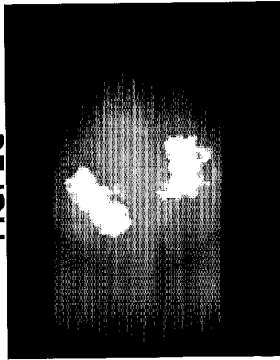


FIG. 2C



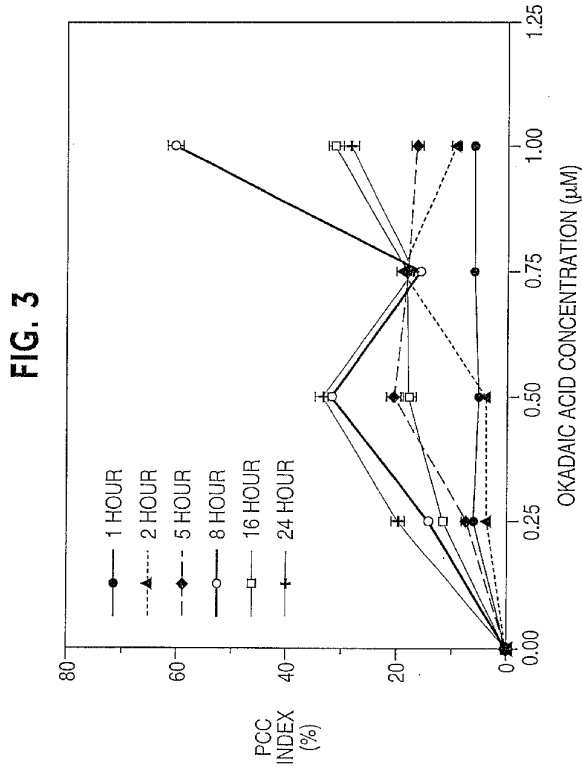


FIG. 4

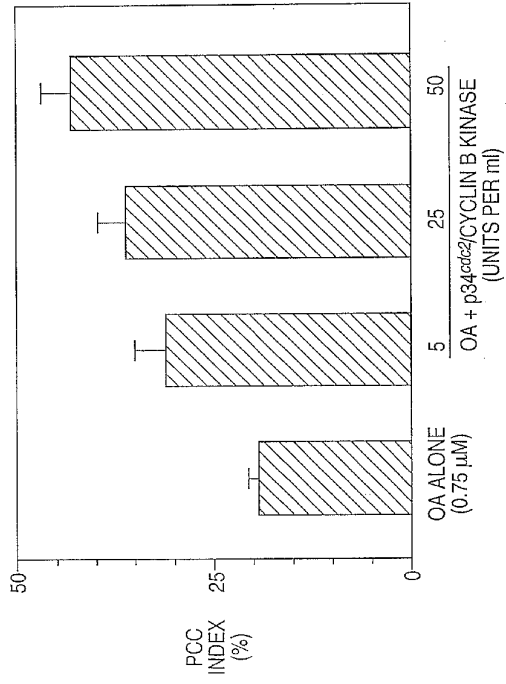
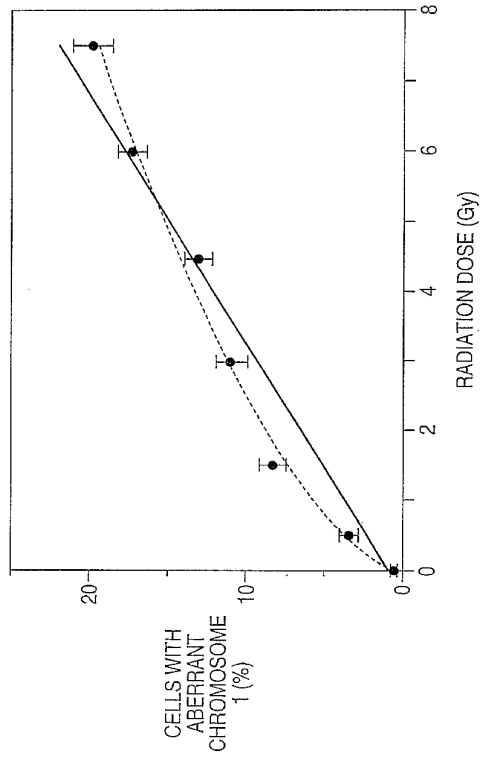
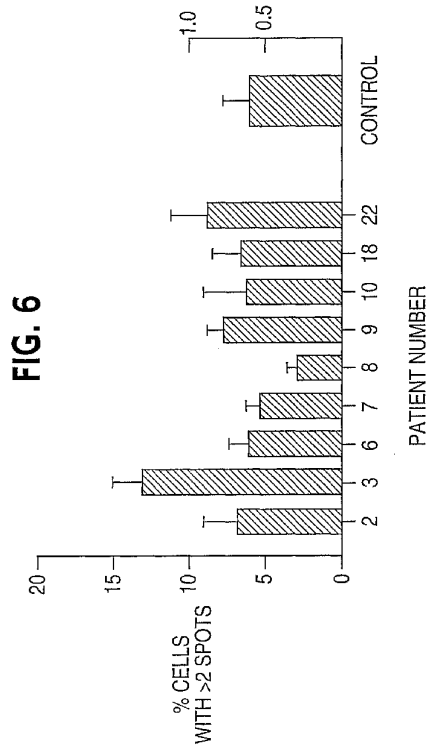


FIG. 5





【 国際調査報告 】

INTERNATIONAL SEARCH REPORT		International application No. PCT/US02/05752
A. CLASSIFICATION OF SUBJECT MATTER		
IPC(7) : C12N 9/00; C12Q 1/00, 1/68; C07H 15/24 US CL : 435/375, 376, 183, 40.5, 29, 6, 536/18.1 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/375, 376, 183, 40.5, 29, 6; 536/18.1		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Continuation Sheet		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PRASANNA, P.G.S. et al. Induction of premature chromosome condensation by a phosphatase inhibitor and a protein kinase in unstimulated human peripheral blood lymphocytes: a simple and rapid technique to study chromosome aberrations using specific whole-chromosome DNA hybridization probes for biological dosimetry. Mutation Res. March 2000, Vol 466, No. 2, pages 131-141, see entire document.	1-6, 8, 9, 16, 17, 19, 21-26, 28-30, 34-43, 45
Y	US 5,789,164A (GOTOH) 04 August 1998 (04.08.1998), see entire document.	7, 10-15, 18, 27, 31-33, 44
Y	PRASANNA, P.G.S. et al. Premature chromosome condensation assay for biodosimetry: studies with fission-neutrons. Health Phys. 1997, Vol. 72, pages 594-600, see entire document.	1-45
Y,P	BERWICK, M. et al. Mutagen sensitivity as an indicator of soft tissue sarcoma risk. Environ. Mol. Mutagen. 19 October 2001, Vol 38 No. 2-3, pages 223-226, see entire document.	10-15
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents:		
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is considered with one or more other such documents, such combination being obvious to a person skilled in the art	
"O" document referring to an oral disclosure, use, exhibition or other means	"Z" document member of the same patent family	
"P" document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 25 July 2002 (25.07.2002)	Date of mailing of the international search report 18 SEP 2002	
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCH Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized Officer Daniel M Sullivan Telephone No. 703-308-0196	

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INTERNATIONAL SEARCH REPORT		PCT/US02/05752
C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KANDA, R. Phosphatase inhibitors and premature chromosome condensation in human peripheral lymphocytes at different cell-cycle phases. <i>Somatic Cell and Molecular Genetics</i> 1999, Vol. 25 No. 1, pages 1-8, see entire document.	1-45
Y	DROPKIN, R.H. et al. Effects on mouse embryos of in utero exposure to saccharin: teratogenic and chromosome effects. <i>Arch. Toxicol.</i> 1985, Vol. 56 No. 4, pages 283-287, see entire document.	10-15, 16-20
Y	STEINMANN, K.E. et al. Chemically induced premature mitosis: differential response in rodent and human cells and the relationship to cyclin B synthesis and p24cdc2/cyclin B complex formation. <i>Proc. Natl. Acad. Sci. USA</i> , August 1991, Vol. 88, pages 6843-6847, see entire document.	1-45
Y	ZHANG, L. Benzene increases aneuploidy in the lymphocytes of exposed workers: a comparison of data obtained by fluorescence in situ hybridization in interphase and metaphase cells. <i>Environ. Mol. Mutagen.</i> 1999, Vol. 34, pages 260-268, see entire document.	16-20, 25-37
Y	COCO-MARTIN, J.M. et al. Detection of radiation-induced chromosome aberrations using fluorescence in situ hybridization in drug-induced premature chromosome condensations of tumour cell lines with different radiosensitivities. <i>Int. J. Radiat. Biol.</i> 1997, Vol. 71, No. 3, 265-273, see entire document.	1-45

INTERNATIONAL SEARCH REPORT

PCT/US02/05752

Continuation of B. FIELDS SEARCHED Item 3:
MEDLINE, CAPLUS BIGSIS, AFS: PREMATURE CHROMOSOME CONDENSATION, CYCLIN KINASE, CYCLIN B,
P34CDC27, PHOSPHATASE INHIBIT, CHROMOSOME SPREAD, METAPHASE SPREAD, CHROMOSOME PAINT,
FLUORESCENCE IN SITU HYBRIDIZATION

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(81) 指定国 AP(GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZM,ZW),EA(AM,AZ,BY,KG,KZ,MD,RU,TJ,TM),EP(AT, BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE,TR),OA(BF,BJ,CF,CG,CI,CM,GA,GN,GQ,GW,ML,MR,NE,SN, TD,TG),AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CO,CR,CU,CZ,DE,DK,DM,DZ,EC,EE,ES,FI,GB,GD,GE, GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,MZ,NO,NZ,OM,PH,P L,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TN,TR,TT,TZ,UA,UG,US,UZ,VN,YU,ZA,ZM,ZW

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专利名称(译)	诱导早熟染色体凝聚的材料和方法		
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申请号	JP2002568699	申请日	2002-02-28
[标]申请(专利权)人(译)	亨利M.杰克逊基金会		
申请(专利权)人(译)	亨利中号..杰克逊基金会		
[标]发明人	プラサナパタジェジーエス ブレイクラーウィリアムエフ		
发明人	プラサナ, パタジェジー.エス. ブレイクラー, ウィリアム, エフ.		
IPC分类号	G01N33/53 C12N5/06 C12N15/09 C12Q1/02 C12Q1/48 C12Q1/68 C12Q1/6841 C12R1/91 G01N33/566 G01N33/68		
CPC分类号	G01N33/6875 C12Q1/485 C12Q1/6841 G01N2500/00		
FI分类号	C12N5/00.E C12Q1/02 C12Q1/68.A G01N33/53.M G01N33/566 C12N15/00.A C12R1/91		
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代理人(译)	鈴木康仁		
优先权	60/271743 2001-02-28 US		
外部链接	Espacenet		

摘要(译)

本发明使用的未刺激的细胞中，为研究染色体异常的简单和快速的方法。过早染色体缩合 (PCC) 通过在有丝分裂促进因子的存在下培养的非刺激的细胞诱导。本发明的方法涉及与细胞刺激或有丝分裂细胞的融合，这是快速相比于现有技术的方法。通过本发明，许多类型的细胞遗传学分析，特别是在原位杂交的方法产生的冷凝染色体 - 可以与化探针和染色体涂染被使用。这一技术可以应用到辐射照射的生物剂量包括均匀系统性低LET (线性能量转移) 曝光 (统一全身低线性能量转移曝光)。

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G01N 33/53	G01N 33/53	M	
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		審査請求 未請求 予備審査請求 有 (全 101 頁) 最終頁	
(21) 出願番号	特願2002-568699 (P2002-568699)	(71) 出願人	501051125
(66) (22) 出願日	平成14年2月28日 (2002. 2. 28)		ザ・ヘンリー・エム・ジャクソン
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(87) 国際公開番号	W02002/068603		ックヴィル、ロックヴィル、バイ
(87) 国際公開日	平成14年9月6日 (2002. 9. 6)		401 スウィート600
(31) 優先権主張番号	60/271, 743	(74) 代理人	100094318
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