

(19) 日本国特許庁(JP)

(12) 公表特許公報(A)

(11) 特許出願公表番号

特表2005-503753

(P2005-503753A)

(43) 公表日 平成17年2月10日(2005.2.10)

(51) Int. Cl. <sup>7</sup>	F I	テーマコード (参考)
C 1 2 N 15/09	C 1 2 N 15/00	4 B O 2 4
GO 1 N 33/53	GO 1 N 33/53	M
GO 1 N 33/532	GO 1 N 33/532	B
GO 1 N 33/569	GO 1 N 33/569	L

審査請求 未請求 予備審査請求 有 (全 240 頁)

(21) 出願番号	特願2002-527314 (P2002-527314)	(71) 出願人	599075070 ペンタナ・メディカル・システムズ・インコーポレーテッド アメリカ合衆国アリゾナ州85737トウーソン・イノベーションパークドライブ1910
(86) (22) 出願日	平成13年9月6日 (2001.9.6)	(74) 代理人	100060782 弁理士 小田島 平吉
(85) 翻訳文提出日	平成15年3月14日 (2003.3.14)	(72) 発明者	ウターモーレン, ジョセフ・ジー アメリカ合衆国アリゾナ州85711トウーソン・イーストナインティーンズストリート5357
(86) 国際出願番号	PCT/US2001/028014		
(87) 国際公開番号	W02002/022874		
(87) 国際公開日	平成14年3月21日 (2002.3.21)		
(31) 優先権主張番号	60/233, 177		
(32) 優先日	平成12年9月15日 (2000.9.15)		
(33) 優先権主張国	米国 (US)		
(81) 指定国	EP (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), AU, CA, JP, MX, US		

最終頁に続く

(54) 【発明の名称】 イン・サイチュー解析のためのオリゴヌクレオチドプローブおよびタンパク質を標識するためのオリゴヌクレオチド配列式

(57) 【要約】

本発明は、細胞もしくは組織サンプル中の複数の核酸標的遺伝子または抗原を検出または局在化するためのオリゴヌクレオチドプローブおよびオリゴヌクレオチドプローブコレクションおよびタンパク質標識を提供する。具体的には、本発明は、各プローブが、配列式 (CTATTT)<sub>n</sub>、(AAAATAG)<sub>n</sub>、または(TTTTATC)<sub>n</sub>もしくは(GATAAAA)<sub>n</sub> [式中、すべての場合「n」は1に等しいかそれを超える]をもつ標識ドメインを有する場合のイン・サイチューハイブリダイゼーション解析における使用のためのオリゴヌクレオチドプローブのコレクションを提供する。本発明は、細胞もしくは組織サンプル中の特定の核酸標的遺伝子を検出または局在化するためのオリゴヌクレオチドプローブのコレクションもしくは「カクテル」を提供する。カクテルは、次のものを検出するために有用である： 遺伝子 (配列番号：1-16を含めて)； 遺伝子 (配列番号：501-509, 511-513, および515)； CMV (サイトメガロウイルス) 遺伝子 (配列番号：221-241を含めて)； EBER (Epstein

Oligonucleotide Probe Design

Common Signal Detection Region Unique Target Detection Region  
5'-[Common Signal Detection Region]-[Unique Target Detection Region]-3'

Alu301 5'-CTATTTTCTATTTTCTATTTTCTATTTTCTATTTTCT-CGAGGCGGGCGGATCACCTGAGGTC-3'

Alu302 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT-CGGGAGCGGAGGTTGCAGTGAGCC-3'

## 【特許請求の範囲】

## 【請求項 1】

配列 ( C T A T T T )<sub>n</sub> およびその補体 ( A A A A T A G )<sub>n</sub> [ 式中、「n」は少なくとも 1 である ] を含んでなるオリゴヌクレオチド標識ドメイン。

## 【請求項 2】

レポーター分子もしくはハプテン分子により検出可能に標識された請求項 1 のオリゴヌクレオチド標識ドメイン。

## 【請求項 3】

ハプテンが O B E A リンカーを介してシトシンの N 4 窒素に結合されたフルオレセインである、請求項 2 のオリゴヌクレオチド標識ドメイン。

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## 【請求項 4】

レポーター分子が発蛍光団である、請求項 1 のオリゴヌクレオチド標識ドメイン。

## 【請求項 5】

発蛍光団が 7 モル % を超える密度において存在する、請求項 1 のオリゴヌクレオチド標識ドメイン。

## 【請求項 6】

標識ドメインが配列 T C ( T T T T A T C )<sub>n</sub> ( またはその相補的式 ) を有する、請求項 1 のオリゴヌクレオチド標識ドメイン。

## 【請求項 7】

配列が配列番号 : 5 8 である、請求項 1 のオリゴヌクレオチド標識ドメイン。

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## 【請求項 8】

少なくとも 7 モル % のシトシンが O B E A リンカーによって検出可能な部分に結合されている、請求項 2 のオリゴヌクレオチド標識ドメイン。

## 【請求項 9】

少なくとも 2 つの別個の機能性ドメイン、請求項 2 の標識ドメインを含んでなる第 1 のドメイン、および遺伝子に特異的な標的配列を含んでなる第 2 のドメインを有するオリゴヌクレオチドプローブ。

## 【請求項 10】

標識ドメインがオリゴヌクレオチドプローブの 5 ' 末端に配置され、そして遺伝子に特異的な標的配列が標識ドメインに対して 3 ' に存在している、請求項 9 のオリゴヌクレオチドプローブ。

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## 【請求項 11】

標識ドメインがオリゴヌクレオチドプローブの 3 ' 末端に配置され、そして遺伝子に特異的な標的配列が標識ドメインに対して 5 ' に存在している、請求項 9 のオリゴヌクレオチドプローブ。

## 【請求項 12】

3 つの別個の機能性ドメイン、請求項 2 の標識ドメインを含んでなる第 1 のドメイン、遺伝子に特異的な標的配列を含んでなる第 2 のドメイン、およびその他の標識ドメインを含んでなる第 3 のドメインを有し、該第 2 のドメインが該第 1 と該第 3 のドメインとの間に配置されているオリゴヌクレオチドプローブ。

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## 【請求項 13】

プローブが本質的に配列番号 : 4 0 1 - 4 1 6 を含めてなる群から選ばれる、免疫グロブリン軽鎖 m R N A または対応する異核 ( h e t e r e o n u c l e a r ) R N A を検出するためのプローブセット。

## 【請求項 14】

プローブが本質的に配列番号 : 5 0 1 - 5 0 9 , 5 1 1 - 5 1 3 、および 5 1 5 からなる群から選ばれる免疫グロブリン軽鎖 m R N A または対応する異核 R N A を検出するためのプローブセット。

## 【請求項 15】

プローブが本質的に配列番号 : 2 2 1 - 2 4 1 からなる群から選ばれるサイトメガロウイ

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ルス (CMV) 即時型 RNA および / または対応する mRNA を検出するためのプローブセット。

【請求項 16】

プローブが本質的に配列番号：51 - 54 からなる群から選ばれるエプスタイン・バーウイルス (EBV) の初期 RNA、RNA 1 および RNA 2、(EBER) を検出するためのプローブセット。

【請求項 17】

プローブが本質的に配列番号：301 および 302 からなる群から選ばれるヒト Alu 反復サテライトゲノム DNA 配列を検出するためのプローブセット。

【発明の詳細な説明】

【0001】

発明の背景

1. 発明の分野

本発明は、細胞もしくは組織サンプル中の核酸遺伝子標的を検出または局在化するためのオリゴヌクレオチドプローブおよびオリゴヌクレオチドプローブのコレクションに関する。特に、本発明はオリゴプローブのコレクションに関する。

【0002】

2. 発明の背景

イン・サイチュ解析はイン・サイチュのハイブリダイゼーションおよび免疫組織化学を含む。イン・サイチュハイブリダイゼーション (ISH) は、細胞もしくは組織サンプル中の標的とする核酸標的遺伝子を検出または局在化するために、標的遺伝子配列もしくは転写物に対してアンチセンスである標識した DNA もしくは RNA プローブ分子を用いる。ISH は、発生生物学、細胞生物学および分子生物学を含む、多数の生物医療分野において有用な道具であることが分かっている。ISH は、例えば、遺伝障害を診断したり、遺伝子をマッピングしたり、遺伝子発現を研究したり、そして標的遺伝子発現の部位を局在化するために使用されてきた。

【0003】

典型的には、ISH は、ガラススライド上に固定化した細胞もしくは組織サンプルを、細胞もしくは組織サンプル中の特定の標的遺伝子に特異的にハイブリダイズすることができる標識した核酸プローブに接触させることによって実施される (In Situ Hybridization: Medical Applications (G. R. Coulton and J. de Belleruche, eds., Kluwer Academic Publishers, 1992); In Situ Hybridization: In Neurobiology; Advances in Methodology (J. H. Eberwine, K. L. Valentino, and J. D. Barchas, eds., Oxford University Press, 1994); In Situ Hybridization: A Practical Approach (D. G. Wilkinson, ed., Oxford University Press, 1992))。細胞もしくは組織サンプル中の核酸への標識したプローブ分子のハイブリダイゼーションは、次に、例えば、放射能に基づく直接検出法、蛍光に基づく直接検出法、または BrdU のようなハプテンに結合している蛍光標識したタンパク質、プローブ中に組み入れられたジゴキシゲニン標識もしくはピオチン標識したヌクレオチドの結合に基づく間接検出法を用いて検出することができる。ハプテンに基づく方法は、さらに、結合性タンパク質 - 酵素複合体、例えば抗体 - 酵素複合体によって結合されるそれらの分子および比色法に基づく検出化学を含めるように拡張された。さらに、いくつかの標的遺伝子が、複数の異なる核酸タグで標識された複数の核酸プローブに細胞もしくは組織サンプルを接触させることによって同時に解析することができる。例えば、複数の核酸プローブが、異なる発光波長をもつ複数の蛍光化合物により標識することができ、それによって単一の標的細胞もしくは組織サンプルにおいて 1 段階で実施される同時多重発色分析を可能にする。

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## 【0004】

オリゴヌクレオチドプローブ中への標識したヌクレオチドの組み入れに伴う重要な問題は、通常、ヌクレオチドに結合している複合部分が、ワトソン・クリック塩基対の形成を妨害し、その結果その標的へのプローブのハイブリダイゼーションにネガティブに影響することである。このことは、非置換シトシン（1級アミン）と形成される自然のG-C結合に較べて、立体障害および2級アミンの低い反応性状態への予期されるシフト（N4標識したシトシンに見られるような）のために、N4-置換されたシトシンヌクレオチドを介して結合された標識の使用に関して見られた。小さいヌクレオチド（25～50塩基）における何らかの小変化またはG-C結合の妨害は、意図した標的配列とハイブリダイズするこれらのオリゴの能力を低下させることがある。

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## 【0005】

オリゴヌクレオチド中に標識したヌクレオチドを組み入れるために適当なプローブ設計を開発する技術上の必要性が残っている。本発明者らは、数種の人工配列が、プローブ標識のための効果的に機能する代替物であり、そしてまた細胞もしくは組織サンプル中の核酸標的遺伝子を検出または局在化するために、単独でも、また複合したオリゴヌクレオチドプローブ混合物においても働くことを例証する。プローブコレクションのためのそのような遺伝子配列の開発および標識戦略は、医療、遺伝子および分子生物学技術における広い応用性を有する。

## 【0006】

標識化学によるこの妨害およびハイブリダイゼーションストリンジェンシーおよび動力学が、本明細書において、少なくとも2つの別個の機能性ドメイン、すなわち遺伝子特異的であり、塩基対形成に必要である1つのドメインもしくは配列、およびスペーシングヌクレオチドと標識したヌクレオチドからなる人工的な非特異的配列（サンプルのゲノムに関して）である第2ドメインを有するオリゴを設計することによって解決される。これらの要素は、これらの標識ヌクレオチドがタンパク質（免疫グロブリンもしくはアビジン）を結合するためにハプテンとして一層作用しやすく、したがって遺伝子に特異的なドメインにおいてワトソン・クリック塩基対を妨害しないように置かれる。

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## 【0007】

## 発明の概要

本発明は、細胞もしくは組織サンプル中の核酸標的遺伝子を検出または局在化するために、オリゴヌクレオチドプローブ中に標識を組み入れるための新規戦略および標識されたオリゴヌクレオチドプローブのコレクションを提供する。特に、本発明は、イン・サイチュハイブリダイゼーション解析における使用のために、オリゴヌクレオチドプローブのコレクション中に組み入れることができるそのような配列の反復ポリマーを作成するための配列式を用いる非遺伝子特異的配列に関する。さらに、配列式に基づく標識された合成オリゴヌクレオチドポリマーを使用することは、結合性タンパク質、すなわち免疫グロブリンに複合された場合には、免疫組織化学的解析において使用されるそのようなタンパク質を標識するための非常に効果的な、そして制御される方法である。本発明は、細胞もしくは組織サンプル中の特定核酸標的遺伝子を検出または局在化するためのオリゴヌクレオチドプローブのコレクションもしくは「カクテル」を提供する。カクテルは、次のものを検出するために有用である： 遺伝子（配列番号：1-16を含めて）； 遺伝子（配列番号：501-509, 511-513, および515）；CMV（サイトメガロウイルス）遺伝子（配列番号：221-241を含めて）；EBER（Epstein-Barr初期RNA）遺伝子（配列番号：51-54を含めて）；Alu（配列番号：55-56）；ポリA（配列番号：57）；および検出テール（配列番号：330）。

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## 【0008】

本発明は、配列 $(CTATTT)_n$  およびその補体 $(AAAATAG)_n$  [式中、「n」は少なくとも1である]を含んでなるオリゴヌクレオチド標識ドメインに向けられる。

## 【0009】

また、本発明は、少なくとも2つの別個の機能性ドメイン、請求項2の標識ドメインを含

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んでなる第1のドメイン、および遺伝子に特異的な標的配列を含んでなる第2のドメインを有するオリゴヌクレオチドプローブに向けられる。

【0010】

また、本発明は、プローブが本質的に配列番号：401 - 416を含めてなる群から選ばれる免疫グロブリン軽鎖mRNAまたは対応する異核(heteronuclear)RNAを検出するためのプローブセットに向けられる。

【0011】

また、本発明は、プローブが本質的に配列番号：501 - 509, 511 - 513、および515からなる群から選ばれる免疫グロブリン軽鎖mRNAまたは対応する異核RNAを検出するためのプローブセットに向けられる。

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【0012】

また、本発明は、プローブが本質的に配列番号：221 - 241からなる群から選ばれるサイトメガロウイルス(CMV)即時型RNAおよび/または対応するmRNAを検出するためのプローブセットに向けられる。

【0013】

また、本発明は、プローブが本質的に配列番号：51 ~ 54からなる群から選ばれるEpstein-Barrウイルス(EBV)初期RNA、RNA1およびRNA2、(EBER)を検出するためのプローブセットに向けられる。

【0014】

また、本発明は、プローブが本質的に配列番号：301および302からなる群から選ばれるヒトAlu反復サテライトゲノムDNA配列を検出するためのプローブセットに向けられる。

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【0015】

本発明の特定の好適な実施態様は、以下のある好適な実施態様および請求項のより詳細な記述から明らかになるであろう。

【0016】

図1は、2ドメインプローブ設計の遺伝子プローブ構造を図解している。これは、次の実施例において記述される遺伝子特異的なカクテルにおけるプローブのために使用されるオリゴヌクレオチドの設計である。各プローブは、2つのドメイン：5'標識ドメインおよび3'標的遺伝子の標的遺伝子に特異的なドメインからなる。標識ドメインは、この特異配列(C T A T T T)<sub>n</sub>からなり、この場合、各シトシンは、発蛍光団もしくはシトシン-ハプテン複合体により標識されてもよく、この実施態様ではハプテンはフルオレセインである。この図は、具体的には、301(配列番号：55)および302(配列番号：56)プローブについての核酸配列を示し、これらの各々は、ヒト反復Alu配列に対応する標的遺伝子の遺伝子特異的なドメインおよびフルオレセインハプテンを有する標識ドメインを保持する。

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【0017】

図2は、標識ドメインを含んでなるプローブ(330プローブ；配列番号：58)を用いるヒト皮膚組織のイン・サイチューハイブリダイゼーション(ISH)解析について得られた結果を説明する。検出可能なシグナルの不在は、これらのISH実施例において使用されたオリゴヌクレオチドに共通する標識ドメインの配列式(C T A T T T)<sub>n</sub>が、それがハイブリダイズしないためにヒト核酸配列とワトソン・クリック塩基対を形成する能力において非特異的で、そして非反応性であることを示している。

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【0018】

図3は、標識ドメインおよびポリd(T)標的遺伝子特異的なドメインを含んでなるプローブ(320プローブ；配列番号：57)を用いるヒト皮膚組織のISH解析について得られた結果を説明する。細胞質に局在される検出可能なシグナルの存在は、このプローブがメッセンジャーRNAのポリアデニル化領域に特異的にハイブリダイズすることができることを示している。

【0019】

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図4 A - 4 Bは、組織サンプルが、イン・サイチューハイブリダイゼーション前にリボヌクレアーゼAにより処理されなかった(A)か、またはイン・サイチューハイブリダイゼーション前にリボヌクレアーゼAにより処理された(B)場合の、320プローブを用いるヒト皮膚組織のISH解析について得られた結果を説明する。(B)における検出可能なシグナルにおける減少は、このプローブがメッセンジャーRNAに共通するポリアデニル化領域に特異的にハイブリダイズすることを示している。

【0020】

図5 A - 5 Bは、ハイブリダイゼーションおよびストリンジェンシー洗浄が室温(A)または37 (B)で実施された場合の、320プローブを用いるヒト脾臓組織のISH解析について得られた結果を説明する。この結果は、より強い発色がより低いストリンジェンシー条件を示すことによって、発色の強度がハイブリダイゼーション条件のストリンジェンシーに関係することを示している。

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【0021】

図6は、320プローブを用いるヒトRaji細胞系のISH解析について得られた結果を説明する。これは、このプローブ設計がまた包埋細胞系ならびに包埋組織を用いて機能することを示している。

【0022】

図7は、301および302プローブからなるプローブコレクションを用いるヒトRaji細胞系のISH解析について得られた結果を説明する。

【0023】

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図8は、301および302プローブからなるプローブコレクションを用いるヒトHT細胞系のISH解析について得られた結果を説明する。

【0024】

図9は、301および302プローブからなるプローブコレクションを用いるラット細胞系のISH解析について得られた結果を説明する。検出可能なシグナルの不在は、このプローブコレクションがヒト核酸配列に特異的であることを示している。

【0025】

図10は、エプスタイン・バーウイルス(EBV) EBER核RNAに対応する標的遺伝子特異的ドメインを保持するプローブ[配列番号: 51 ~ 配列番号: 54 ?]を用いるEBV陰性ヒトHT細胞系のISH解析について得られた結果を説明する。

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【0026】

図11は、EBV EBER1および2核RNAに対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション[配列番号: 51 ~ 配列番号: 54]を用いるヒト脾臓組織のISH解析について得られた結果を説明する。

【0027】

図12は、EBV EBER1および2核RNAに対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション[配列番号: 51 ~ 配列番号: 54]を用いるヒト扁桃組織のISH解析について得られた結果を説明する。

【0028】

図13 A - 13 Bは、EBV EBER1および2核RNAに対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション[配列番号: 51 ~ 配列番号: 54]を用いるヒト脾臓組織のISH解析について得られた結果を説明するが、この場合、組織サンプルは、イン・サイチューハイブリダイゼーション前にリボヌクレアーゼAにより処理されなかった(A)か、またはイン・サイチューハイブリダイゼーション前にリボヌクレアーゼAにより処理された(B)。(B)における検出可能なシグナルにおける減少は、このプローブがヒトEBER1および2核RNAに特異的にハイブリダイズすることを示している。

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【0029】

図14は、ヒト免疫グロブリン 軽鎖mRNAに対応する標的遺伝子特異的ドメインを保持するプローブ[配列番号: 15]を用いる 軽鎖ポジティブヒト扁桃組織のISH解析

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について得られた結果を説明する。

【0030】

図15は、ヒト免疫グロブリン 軽鎖 mRNA に対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション [ 配列番号：2 - 4、配列番号：7 - 12、配列番号：14, 15 ] を用いるリンパ腫組織のISH解析について得られた結果を説明する。(A)のリンパ腫組織は 軽鎖を過発現し、そして(B)の組織は 軽鎖を過発現する。(B)における検出可能なシグナルの不在は、 軽鎖プローブコレクションが 軽鎖 mRNA に特異的であることを示している。

【0031】

図16は、ヒト免疫グロブリン 軽鎖可変部 mRNA に対応する標的遺伝子特異的ドメインを保持するプローブ [ 配列番号：19 - 29 ] を用いる 軽鎖ポジティブヒト扁桃組織のISH解析について得られた結果を説明する。

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【0032】

図17は、ヒト免疫グロブリン 軽鎖 mRNA に対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション [ 配列番号：19 - 29 ] を用いる 軽鎖ポジティブヒトRPMI 8226細胞系のISH解析について得られた結果を説明する。

【0033】

図18A - 18Bは、ヒト免疫グロブリン 軽鎖 mRNA に対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション [ 配列番号：19 - 29 ] を用いるヒト脾臓組織のISH解析について得られた結果を説明する。(A)の組織は 軽鎖を過発現し、そして(B)の組織は 軽鎖を過発現する。(B)における検出可能なシグナルの不在は、 軽鎖プローブコレクションがヒト 軽鎖 mRNA に特異的であることを示している。

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【0034】

図19は、CMV即時型RNAに対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション [ 配列番号：30 - 32、配列番号：34 - 35、配列番号：38、配列番号：50 ] を用いるサイトメガロウイルス(CMV)ポジティブヒト肺組織のISH解析について得られた結果を説明する。[CMV感染細胞]

図20は、CMV即時型mRNAに対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション [ 配列番号：30 - 32、配列番号：34 - 35、配列番号：38、配列番号：50 ] を用いる、CMV即時型RNAの発現がシクロヘキサミドによって誘発されなかったラット9G細胞系のISH解析について得られた結果を説明する。

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【0035】

図21A - 21Bは、CMV即時型RNAに対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション [ 配列番号：30 - 32、配列番号：34 - 35、配列番号：38、配列番号：50 ] を用いる、CMV即時型RNAの発現がシクロヘキサミドによって誘発されたラット9G細胞系のISH解析について得られた結果を説明する。(A)の組織は倍率40Xにおいて示され、そして(B)の組織は倍率20Xにおいて示される。

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【0036】

好適な実施態様の詳細な記述

本発明は、細胞もしくは組織サンプル中の核酸標的遺伝子を検出または局在化するためのオリゴヌクレオチドプローブおよびオリゴヌクレオチドプローブコレクションを提供する。特に、本発明は、イン・サイチューハイブリダイゼーション解析における使用のためのオリゴヌクレオチドプローブのコレクションに関する。

【0037】

より具体的には、本発明は、オリゴヌクレオチドプローブまたはタンパク質に検出可能な部分(標識)を結合するためのヌクレオチドポリマーまたは標識ドメインについての特定

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の配列式の使用に関する。これらの配列もしくはその誘導体の特定の利用は、ハイブリダイゼーション条件の標準ストリンジェンシー下では検出可能なレベルにおいてヒトDNAもしくはRNAにハイブリダイズしないという不活性または非反応性の特性である。これらの標識ドメインまたはポリマーは、イン・サイチューハイブリダイゼーション解析において細胞もしくは組織サンプル中の遺伝子に特異的な配列を検出するためのオリゴヌクレオチドプローブ中への組み入れに有用な遺伝子配列であることが例証された。さらに、この不活性な配列のセットは、免疫組織化学的解析においてハプテンおよび抗原を検出するために、免疫グロブリンもしくは他のタンパク質に標識を結合させるために有用である。

【0038】

本明細書で使用されるように、用語「プローブ」もしくは「オリゴヌクレオチドプローブ」は、相補的核酸標的遺伝子を検出するために使用される核酸分子を指す。 10

【0039】

本明細書で使用されるように、用語「ハイブリダイゼーション」は、相補的核酸配列が接合して二本鎖核酸分子を形成する過程を指す。例えば、放射性もしくは蛍光タグにより標的核酸分子を標識することによって、プローブと標的遺伝子間の相互作用が検出できる。

【0040】

本発明のオリゴヌクレオチドプローブおよびコレクションのオリゴヌクレオチドプローブは慣用の方法を用いて合成される。参照、例えば、Methods in Molecular Biology, Vol 20: Protocols for Oligonucleotides and Analogs 165-89 (S. Agrawal, ed., 1993); Oligonucleotides and Analogues: A Practical Approach 87-108 (F. Eckstein, ed., 1991)。 20

【0041】

本発明の好適な実施態様では、オリゴヌクレオチドプローブは、2つの別個のドメイン：5'（または標識）ドメインおよび3'（または遺伝子に特異的な標的）ドメインを保持する（参照、図1A）。本発明のより好適な実施態様では、オリゴヌクレオチドプローブは、配列（CTATTT）<sub>n</sub> からなる標識ドメインを保持する。また、他の実施態様が本明細書において例証され、これは2つの末端の標識ドメインおよび中央の遺伝子特異的標的ドメインを有する3重ドメイン態様も含む。具体的には、配列番号：125-126がこの標識スキームを表している。なお、標識ドメインのさらなる好適な実施態様は、TC(TTTATC)<sub>n</sub> またはその補体である。この配列は（CTATTT）<sub>n</sub> 標識ドメインと同じくらい独特であると予想される。本発明のオリゴヌクレオチドプローブは、特定の細胞もしくは組織において該プローブと標的核酸との間のハイブリダイゼーションが検出できるように標識される。イン・サイチューハイブリダイゼーション（ISH）解析における使用のために許容される標識は当業者には既知である。そのような標識は、例えば、放射能に基づく直接検出法、蛍光に基づく直接検出法、蛍光に基づく直接検出法と共役されたジゴキシゲン標識もしくはビオチン標識したプローブ、または抗体-酵素に基づく検出法と共役されたジゴキシゲン標識もしくはビオチン標識したプローブを用いて、プローブと標的遺伝子間の相互作用が検出されるのを可能にする。本発明の好適な実施態様では、オリゴヌクレオチドプローブはフルオレセインにより標識される。本発明のより好適な実施態様では、オリゴヌクレオチドプローブは、シトシンヌクレオチドが、直接検出のための発蛍光団、または間接検出のためのハプテンにより標識されてもよい配列（CTATTT）<sub>n</sub> からなる標識ドメインを保持する。いずれにおいても、フルオレセイン-シトシンヌクレオチド複合体およびフルオレセイン分子は、OBEA結合を介してシトシンのN4位に結合される（引用によって本明細書に組み入れられているMishra et al., 米国特許第5,684,142号参照）。好適な実施態様では、標識ドメインに結合される発蛍光団の密度は、標識ドメインのみに対して測定された場合、少なくとも7モル%、好ましくは少なくとも10モル%、もっとも好ましくは少なくとも16モル%である。例えば、プローブ401が考えられる場合（2ドメインプローブ）、そ 40 50

れは、Cがまた標識される3'末端CTを含む30塩基の標識ドメインを含有し、モル%は $5/30 = 16.7$ モル%標識である。プローブ全体では、モル%は8.3である。

【0042】

本発明の若干の実施態様では、いくつかの標的遺伝子が、複数の異なる核酸タグで標識された複数の核酸プローブに細胞もしくは組織サンプルを接触させることによって同時に解析される。例えば、複数の核酸プローブが、異なる発光波長をもつ複数の蛍光化合物により標識でき、それによって単一の標的細胞もしくは組織サンプルにおいて1段階で実施される同時多色分析を可能にする。

【0043】

本発明のオリゴヌクレオチドプローブおよびオリゴヌクレオチドプローブコレクションは、細胞もしくは組織サンプル中の核酸標的遺伝子を検出または局在化するためにISH解析において使用することができる。ISH解析は、例えば、In Situ Hybridization: Medical Applications (G. R. Coulton and J. de Belleruche, eds., Kluwer Academic Publishers, 1992); In Situ Hybridization: In Neurobiology; Advances in Methodology (J. H. Eberwine, K. L. Valentino, and J. D. Baruchas, eds., Oxford University Press, 1994); または In Situ Hybridization: A Practical Approach (D. G. Wilkinson, ed., Oxford University Press, 1992) において記述されるように実施されてもよい。

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【0044】

本発明のプローブおよびプローブコレクションの好適な実施態様は、図1-21および実施例1-2を参照することによってもっとも良く理解される。以下に示す実施例は、本発明の特別な実施態様および種々のその使用の具体的説明である。それらは、説明の目的のためにのみ記述され、そして本発明を限定するものとして解釈されるべきではない。

【0045】

実施例1

プローブコレクションの調製

長さ55~60塩基の多数のオリゴヌクレオチドプローブからなるプローブコレクションが次のように設計された。この実施例では、各オリゴヌクレオチドプローブは、2つの別個のドメイン: 5' (または標識) ドメインおよび3' (または標的遺伝子に特異的) ドメインを保持した (参照、図1)。

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【0046】

この実施態様では、標識ドメインは配列 (CTATTT)<sub>n</sub> からなり、この場合、シトシンヌクレオチドは、フルオレセイン-シトシンヌクレオチド複合体を表し、そしてフルオレセイン分子は、OBEA結合を介してシトシンのN4位に結合される。

【0047】

標的遺伝子に特異的なドメインは、特定核酸標的遺伝子に対して相補的である25-30塩基配列からなる。オリゴヌクレオチドプローブは、ヒト免疫グロブリン 軽鎖可変部 (参照、表1; オリゴヌクレオチドプローブ401-416)、ヒト免疫グロブリン 軽鎖可変部 (オリゴヌクレオチドプローブ501-515)、ヒトサイトメガロウイルス (CMV) 配列 (オリゴヌクレオチドプローブ221-241)、ヒトEpstein-Barrウイルス (EBV) EBER (Epstein-Barr初期RNA) 配列 (オリゴヌクレオチドプローブ100A2, 100C2, 100A1および100B1)、ヒト反復Alu配列 (オリゴヌクレオチドプローブ301および302)、およびポリd (T) (オリゴヌクレオチドプローブ320) に対応する標的遺伝子特異的ドメインを保持するよう設計された。

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【0048】

実施例2

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標識ドメイン設計：A1u反復配列プローブ

A1uヒト反復配列に対するすべて4プローブが使用されて標識ドメイン設計を評価した。プローブ番号301(配列番号:55)、301A(配列番号:116)、301A2/2(配列番号:121)、および301A3/2(配列番号:122)が表1に示される。

## 【0049】

4プローブは、それぞれ、反応液中プローブ1ml当たり濃度100, 75, 50および25ng/mlにおいて評価された。このハイブリダイゼーション解析は、標準プロトコルを用いてマニュアルで実施された。標的、パラフィン包埋細胞系MBA MD468 (Oncor INFORM<sup>TM</sup> Her-2/neu Control Slides, Cat. No. S8100, Level 1, Ventana Medical Systems, Inc., Tucson, AZ)が標的サンプルであり、そして標準キシレン法によってパラフィンを除去することによって処理された。組織は、VentanaのAPKバッファーによる1:2希釈液として、50で12分間VentanaのProtease 1にさらされた。ハイブリダイゼーション反応は、2XSSC/Triton X-100の残留100ul容量に対して、100ulプローブとしてのプローブ希釈液(25%ホルムアミド、5%デキストラン硫酸、2XSSC、1%Triton)の添加により達成された。スライドは85で5分間加熱され、次いで、37で1時間インキュベートされた。標準SSC洗浄が過剰のプローブを除去するために続いた。ハイブリッドはFITCに対する抗体により検出された。マウス抗体は、Ventana Enhanced Alkaline Phosphatase Blue Detection (cat # 760-061)を用いて比色法により検出された。特に指摘しなければ、すべての試薬はVentana Medical Systems, Inc., Tucson, AZから得られた。結果は明視野顕微鏡を用いて発色検出によって観察された。

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## 【0050】

これらの実験の結果は、シグナル強度がプローブに共役されたフルオレセインハプテンの総数の関数であり、そしてシグナルが特定の標識ドメイン設計に属することであった。プローブ分子当たりのフルオレセイン数が大きいほど、より大きいシグナルが観察された。設計の比較およびプローブへのハプテンの配置は、これがシグナルの強度におけるファクターではないことを示した。5フルオレセインを含有した2つのプローブ(301A3/2(配列番号:122)および301(配列番号:55))両方は等しいシグナルを生じた。これらの2つのプローブは、301A2/2、4フルオレセインを有する分離した標識ドメイン設計を有するプローブ、について見られるよりも大きいシグナルを生じた。プローブ301A2/2は、プローブ301A、5'末端に1つの標識ドメイン設計を有し、そして3フルオレセインを有するプローブ、よりも大きいシグナルを生じた。

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## 【0051】

実施例3標識ドメイン設計：EBERプローブ

この実験は、フルオレセインハプテン間のより大きい間隔が、イン・サイチューハイブリダイゼーション解析におけるプローブ検出段階におけるシグナル生成を改善できるか否かを決定するために、2つの標識ドメイン設計および配列を比較した。

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## 【0052】

使用された組織は、シラン+ガラス顕微鏡スライド上に置かれた厚さ4ミクロンの中性バッファーホルマリンパラフィン包埋切片中に固定されたEBV感染ヒト脾臓組織であった。組織切片は、Ventana DISCOVERY<sup>TM</sup> 機器において脱パラフィンされ、続いて温度37でVentanaのProtease 1により6分間消化された。プローブは、Ventana Medical Systems, Inc.の自動ISH染色システム, Discoveryによって調製された後にスライド上に残された等容量の2XSSC/Triton X-100残留容量に対して適用される100ulとして、濃度50ng/mlにおいてハイブリダイゼーションバッファー希釈液に溶解された。プロ

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ープ希釈液は37で6分間スライド上で残留容量と混合され、次いで、溶液は85に加熱され、そして全10分間そこで維持された。次いで、スライドは温度37にされ、そしてその温度で1時間維持された。スライド上のこれらの水性反応のすべては、処理中の水の蒸発損失を防ぐためにLIQUID COVER SLIP<sup>TM</sup>のフィルム下ですべて実施された。ハイブリダイゼーション後の各スライドは、各洗浄間に6分間インキュベーションしつつ2XSSC/Triton溶液で3回洗浄され、スライド容量は約300ul(+/-10%vol)であった。ハイブリッドはFITCに対する抗体により検出された。マウス抗体は、Ventana Enhanced Alkaline Phosphatase Blue Detection (cat#760-061)を用いて比色法により検出された。

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#### 【0053】

この研究のために使用された2つのオリゴヌクレオチドプローブは、プローブ100A1(配列番号:53)および1002A32(配列番号:120)である。これらのプローブの間の2つの差異は標識ドメイン配列と構造であった。配列式(CTATTT)<sub>4</sub>CT(配列番号:58)を有するプローブ100A1の標識ドメインは、遺伝子標的ドメインに対して5'に存在し、OBEAリンカーを介してシトシン残基に結合された5フルオレセインを含有していた。オリゴプローブ1002A32の標識ドメインは(配列番号:125)に類似していた。異なる配列以外に、主要な差異は、シトシン間隔が7塩基離れて比較的近くに存在するオリゴ100A1に較べて、フルオレセイン標識されたシトシンが、10塩基離れて間隔を置かれているということである。Hスコア解析によって推定されるこの比較の結果は、これらのオリゴヌクレオチドがスライド上で生成されたシグナル量に関して同等であることであった。データは、100A2については、3視野のすべてにおいて解析された368細胞ではHスコアは106であり、そしてプローブ1002A32については、3視野において解析された345細胞ではHスコアは109であることであった。Hスコアは、スライド上の総標的的特異的シグナルの相対的比較を得るために、組織切片におけるスコアバックグラウンド対シグナル比に因数分解する、顕微鏡を用いて実施される分光写真解析である。(参照、引用文献Giroud, F. Perrin C, and Simony Lafontaine, J.; Quantitative Immunocytochemistry and Immunohistochemistry. Third Conference of the European Society for Analytical Cellular Pathology, 1994; and AutoCyte Quick Immuno User's Manual, 1998, document number PA-029, Co AutoCyte Inc. Burlington NC2721)。ヒストグラムおよびスコアシートは、各オリゴが比色シグナルを生じる上で等しく有効であったことを示している。このことは、標識ドメインの位置が遺伝子標的配列に対して3プライムであっても5プライムであってもよいか、または遺伝子標的配列が2つの標識ドメイン間に位置していてもよいことを示している。

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#### 【0054】

##### 実施例4

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##### イン・サイチューハイブリダイゼーション

実施例1において調製されたプローブコレクションは、最初に、20%デキストラン硫酸(wt/vol)、50%ホルムアミド(vol/vol)、2XSSC、10mMtris-HCl、5mMEDTAおよび0.05%Brij-35からなる溶液において最終pH7.3において希釈された。次いで、プローブコレクションは2XSSCおよび0.05%TritonX-100からなる溶液の等容量と混合された。

#### 【0055】

ISH解析のためのサンプルは、ホルマリン固定され、パラフィン包埋された細胞もしくは組織サンプルを4μmの切片に切断し、そして切片をガラススライド上に置くことによって調製された。続くサンプルの処理およびISHは、自動装置、例えば、両方とも引用

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によって本明細書に組み入れられている同時所有で同時係属中の米国特許出願第60/076,198号および同第09/259,240号に記述されているDISCOVERY<sup>TM</sup> Automated ISH/IHC Stainer (Ventana Medical Systems, Inc. Tucson, AZ)において実施された。サンプルからパラフィンを除去するために、スライドは水溶液に浸漬され、約20分間加熱され、次いで洗浄された。自動脱パラフィン操作は、両方とも引用によって本明細書に組み入れられている米国特許出願第60/099,018号および同第09/259,240号に一層完全に記述されている。次いで、サンプルはプロテアーゼで処理され、そしてスライドは、85 (RNA標的遺伝子に対するハイブリダイゼーションのため)もしくは90-95 (DNA標的遺伝子に対するハイブリダイゼーションのため)に4~10分間加熱された。

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**【0056】**

ハイブリダイゼーション反応液は、典型的には、10%デキストラン硫酸(wt/vol)、25%ホルムアミド(vol/vol)、2XSSC、5mM Tris、2.5mM EDTA、0.025%Brij-35、Triton X-100、および各個々のプローブ分子25~125ng/mlからなるハイブリダイゼーションバッファーにおいて行われた。ISH反応は37~54において実施された。実施例1において記述されるプローブコレクションを用いるISHでは、ハイブリダイゼーション反応は場合によっては47で1時間実施された(ハイブリダイゼーション反応が場合によっては37で1時間実施されたポリd(T)プローブを除く)。

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**【0057】**

サンプル中の特定の標的遺伝子に対するフルオレセイン標識プローブ分子のハイブリダイゼーションは、一連の結合性タンパク質を用いることによって検出された、すなわち二次抗体検出。しかしながら、結合されたプローブを可視化できるdetect検出法を使用することも同じく可能である。二次検出では、最初に、フルオレセイン標識されたプローブ分子に対向された抗フルオレセインマウスモノクローナル抗体がサンプルに添加された。次に、マウス抗体に対向したビオチン標識ポリクローナルヤギ抗体がサンプルに添加された。最後に、ハイブリダイゼーション反応物は、リン酸5-プロモ-4-クロロ-3-インドリル/ニトロブルーテトラゾリウム(BCIP/NBT)基質を用いて比色法により検出された。この技術は、「二次抗体検出」と呼ばれ、当業者には日常的に行われる。一次および二次抗体は、Ventana Medical Systems, Inc. Tucson, AZを含む多くの販売元から得ることができ、これらはVentana自動染色システム(ES<sup>R</sup>, NexES<sup>R</sup>, DISCOVERY<sup>TM</sup>, およびBENCHMARK<sup>TM</sup>)において使用するために最適化されている。

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**【0058】**

図2-21は、図1において図解される構造モチーフを有する本明細書で開示され、特許請求されるプローブまたはそのようなプローブからなるプローブコレクションを使用して、種々の細胞系もしくは組織サンプルのイン・サイチューハイブリダイゼーション解析について得られた結果を説明している。

**【0059】****【表1】**

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表1

プロンプ ID	配列	配列 ID	
401	5'-CTATTTCTATTTCTATTTCTATTTCT CCAGAGTAGCAGGAGCCCCAGGAGCTGAGC-3'	1	
402	5'-CTATTTCTATTTCTATTTCTATTTCT	GGATGGAGACTGGGTCAACTGGATGTCACA-3'	2
403	5'-CTATTTCTATTTCTATTTCTATTTCT	GCAAGCGATGGTGACTCTGTCTCCTACAGC-3'	3
404	5'-CTATTTCTATTTCTATTTCTATTTCT	TCTGTCCAGATCCACTGCCACTGAACCTT-3'	4
405	5'-CTATTTCTATTTCTATTTCTATTTCT	GCAGCCACAGTTGCTTCATCTGCACCTTG-3'	5
406	5'-CTATTTCTATTTCTATTTCTATTTCT	TTTCAACTGCTCATCAGATGGCGGGAAGAT-3'	6
407	5'-CTATTTCTATTTCTATTTCTATTTCT	AAGTTATTCAGCAGGCACACAACAGAGGCA-3'	7
408	5'-CTATTTCTATTTCTATTTCTATTTCT	GGCGTTATCCACCTTCCACTGTACTTTGGC-3'	8
409	5'-CTATTTCTATTTCTATTTCTATTTCT	TAGGTGCTGTCCTTGCTGCTGCTGCTGTG-3'	9
410	5'-CTATTTCTATTTCTATTTCTATTTCT	GTAGTCTGCTTTGCTCAGCGTCAGGGTGCT-3'	10
411	5'-CTATTTCTATTTCTATTTCTATTTCT	GATGGGTGACTTCGCAGGCGTAGACTTTGT-3'	11
412	5'-CTATTTCTATTTCTATTTCTATTTCT	CTCTCCCCTGTTGAAGCTCTTTGTGACGGG-3'	12
413	5'-CTATTTCTATTTCTATTTCTATTTCT	TGGAAGTGAAGGAGCAGGTGGGGGCACTTCT-3'	13
414	5'-CTATTTCTATTTCTATTTCTATTTCT	GAAAAAGGGTCAGAGGCCAAGGATGGGAG-3'	14
415	5'-CTATTTCTATTTCTATTTCTATTTCT	AGATGAGCTGGAGGACCGCAATAGGGGTAG-3'	15

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【 0 0 6 0 】

【 表 2 】

416	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>GCATAATTAAGCCAAGGAGGAGGAGGGG</u> -3'	16
501	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>CCTGAGTGAGGAGGGTGAGGAGCAGCAGAG</u> -3'	17
502	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>AGACCCAGACACGGAGGCAGGCTGAGTCAG</u> -3'	18
503	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>TGTTGGTTCAGTGCAGGAGATGGTGATCG</u> -3'	19
504	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>TAAATCARGATTTGGGGGCTTTGCCCTGGG</u> -3'	20
505	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>TGTTGCCAGACTTGGAGCCAGAGAAGCGAT</u> -3'	21
506	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>AATAATCAGCCTCGTCCTCAGCCTGGAGCC</u> -3'	22
507	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>GGTCCTCCGCCGAAAACCACAGTGAAGT</u> -3'	23
508	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>TTATGAGACACACCAGTGTGGCCTTGTGG</u> -3'	24
509	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>CTGCTCAGGCGTCAGGCTCAGATAGCTGCT</u> -3'	25
511	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>ATGCGTGACCTGGCAGCTGTAGCTTCTGTG</u> -3'	26
512	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>ATTCTGTAGGGGCCACTGTCCTCCACGG</u> -3'	27
513	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>CCTCCCCTGGGATCCCTGCAGCTCTAGTCTC</u> -3'	28
515	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>TGAGGGTTTATTGAGTGCAGGAGAGAAGGGC</u> -3'	29
221	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>GGAGGTCAAACACAGCGTGGATGGCG</u> -3'	30
222	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>GAGGCTGGATCCGTCCCGGTGCTCTI</u> -3'	31
223	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>AATCCGCGTTCCAATGCACCGTTCC</u> -3'	32
224	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>TAAAACTGCGGGCACTGGGACCG</u> -3'	33
225	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>ACCCGAGATTCGCGTGGAGATCCCA</u> -3'	34
226	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>GAGCAAGGAGCTGCCGAGCGACCAT</u> -3'	35
227	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>ACACTGGTGGTGGTGGGCATCGTGC</u> -3'	36
228	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>TTCCAAATGCGTCAGCGGTGCAAGC</u> -3'	37
229	5'-CTATTTCTATTTCTATTTCTATTTCT	<u>AGCTGCCTGCATCTTCTTCTGCCGC</u> -3'	38

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【 0 0 6 1 】

【 表 3 】

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238	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>TCTCAGAGGATCGGCCCCAGAAATG</u> -3'	47
239	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>CCTCATCTGACTCCTCGGCGATGGC</u> -3'	48
240	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>CGGGTACAGGGGACTCTGGGGGTGA</u> -3'	49
241	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>GGTGGGTGCTCTTGCCTCCAGAGG</u> -3'	50
100A2	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>GACCTCGGGTCGGTAGCACCGCACT</u> -3'	51
100C2	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>GGAAGCCTCTCTTCCTCCCCCGG</u> -3'	52
100A1	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>CCACAGACACCGTCCTCACCACCGG</u> -3'	53
100B1	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>GGCTACAGCCACACACGTCTCCTCC</u> -3'	54
301	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>CGAGGCGGGCGGATCACCTGAGGTC</u> -3'	55
302	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>CGGGAGGCGGAGGTGCAGTGAGCC</u> -3'	56
320	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT <u>TTTTTTTTTTTTTTTTTTTTTTTTTT</u> -3'	57
301A	5'-CTATTTTCTATTTTCTTTT <u>CGAGGCGGGCGGATCACCTGAGGTC</u> -3'	116
302C	5'-CTATTTTCTATTTTCTTTT <u>CGGGAGGCGGAGGTGCAGTGAGCC</u> -3'	117
302A4	5'-CTATTTTATACTTTATATTTTATATTTATCT <u>CGGGAGGCGGAGGTGCAGTGAGCC</u> -3'	118
302A3/2	5'-CTATTTTATACTTTATATTTTCT <u>CGGGAGGCGGAGGTGCAGTGAGCC</u> ACTATTTTATACTT-3	119
1002A32	5'-CTATTTTATACTTTATATTTTCT <u>GACCTCGGGTCGGTAGCACCGCAC</u> TACTATTTTATACTT-3'	120
301A2/2	5'-CTATTTTCTT <u>CGAGGCGGGCGGATCACCTGAGGTC</u> TTCTTTTATCTT-3	121
301A3/2	5'-CTATTTTATACTTTATATTTTCT <u>CGAGGCGGGCGGATCACCTGAGGTC</u> ACTATTTTATACTT-3'	122

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【 0 0 6 2 】

【 表 4 】

表2

プローブ ID	配列	配列 ID
	5'-CTATTTTCTATTTTCTTTT	123
	5'-CTATTTTATACTTTATATTTTATATTTTATCT	124
330	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT	58
	5'-CTATTTTATACTTTATATTTTCT.....ACTATTTTATACTT-3	125
	5'-CTATTTTCTT.....TTCTTTTATCTT-3	126

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【 0 0 6 3 】

前記の開示は、ある特定の発明の実施態様を強調しており、そしてあらゆるその改変もしくは代替等価物は、添付される請求項において記述される本発明の精神および範囲内にあることが理解されるべきである。

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【 図面の簡単な説明 】

【 図 1 】

2ドメインプローブ設計の遺伝子プローブ構造を図解する。これは、次の実施例において記述される遺伝子特異的カクテルにおけるプローブのために使用されるオリゴヌクレオチドの設計である。各プローブは、2つのドメイン：5'標識ドメインおよび3'標的遺伝子の標的遺伝子に特異的なドメインからなる。標識ドメインは、この特異配列（CTATTT）<sub>n</sub>からなり、この場合、シトシンヌクレオチドは、シトシン-ハプテン複合体であり、この実施例ではハプテンはフルオレセインである。この図は、具体的には、301（配列番号：55）および302（配列番号：56）プローブについての核酸配列を示し、これらの各々は、ヒト反復Alu配列に対応する標的遺伝子の遺伝子特異的ドメインお

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よびフルオレセインハプテンを有する標識ドメインを保持する。

【図 2】

標識ドメインを含んでなるプローブ (330 プローブ; 配列番号: 58) を用いるヒト皮膚組織のイン・サイチュールハイブリダイゼーション (ISH) 解析について得られた結果を説明する。検出可能なシグナルの不在は、これらの ISH 実施例において使用されたオリゴヌクレオチドに共通する標識ドメインの配列式 (CTATTT)<sub>n</sub> が、それがハイブリダイズしないためにヒト核酸配列とワトソン・クリック塩基対を形成する能力において非特異的で、そして非反応性であることを示している。

【図 3】

標識ドメインおよびポリ d (T) 標的遺伝子特異的ドメインを含んでなるプローブ (320 プローブ; 配列番号: 57) を用いるヒト皮膚組織の ISH 解析について得られた結果を説明する。細胞質に局在される検出可能なシグナルの存在は、このプローブがメッセンジャー RNA のポリアデニル化領域に特異的にハイブリダイズできることを示している。 10

【図 4】

組織サンプルが、イン・サイチュールハイブリダイゼーション前にリボヌクレアーゼ A により処理されなかった (A) か、またはイン・サイチュールハイブリダイゼーション前にリボヌクレアーゼ A により処理された (B) 場合の、320 プローブを用いるヒト皮膚組織の ISH 解析について得られた結果を説明する。(B) における検出可能なシグナルにおける減少は、このプローブがメッセンジャー RNA に共通するポリアデニル化領域に特異的にハイブリダイズすることを示している。 20

【図 5】

ハイブリダイゼーションおよびストリンジェンシー洗浄が室温 (A) または 37 (B) で実施された場合の、320 プローブを用いるヒト脾臓組織の ISH 解析について得られた結果を説明する。この結果は、より強い発色がより低いストリンジェント条件を示すことによって、発色の強度がハイブリダイゼーション条件のストリンジェンシーに関係することを示している。

【図 6】

320 プローブを用いるヒト Raji 細胞系の ISH 解析について得られた結果を説明する。これは、このプローブ設計がまた包埋細胞系ならびに包埋組織を用いて機能することを示している。 30

【図 7】

301 および 302 プローブからなるプローブコレクションを用いるヒト Raji 細胞系の ISH 解析について得られた結果を説明する。

【図 8】

301 および 302 プローブからなるプローブコレクションを用いるヒト HT 細胞系の ISH 解析について得られた結果を説明する。

【図 9】

301 および 302 プローブからなるプローブコレクションを用いるラット細胞系の ISH 解析について得られた結果を説明する。検出可能なシグナルの不在は、このプローブコレクションがヒト核酸配列に特異的であることを示している。 40

【図 10】

Epsstein-Barr ウイルス (EBV) EB ER 核 RNA に対応する標的遺伝子特異的ドメインを保持するプローブ [配列番号: 51 ~ 配列番号: 54] を用いる EBV 陰性ヒト HT 細胞系の ISH 解析について得られた結果を説明する。

【図 11】

EBV EB ER 1 および 2 核 RNA に対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション [配列番号: 51 ~ 配列番号: 54] を用いるヒト脾臓組織の ISH 解析について得られた結果を説明する。

【図 12】

EBV EB ER 1 および 2 核 RNA に対応する標的遺伝子特異的ドメインを保持するプ 50

ローブからなるプローブコレクション [ 配列番号 : 5 1 ~ 配列番号 : 5 4 ] を用いるヒト扁桃組織のISH解析について得られた結果を説明する。

【図13】

EBV EBER1および2核RNAに対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション [ 配列番号 : 5 1 ~ 配列番号 : 5 4 ] を用いるヒト脾臓組織のISH解析について得られた結果を説明するが、この場合、組織サンプルは、イン・サイチュハイブリダイゼーション前にリボヌクレアーゼAにより処理されなかった(A)か、またはイン・サイチュハイブリダイゼーション前にリボヌクレアーゼAにより処理された(B)。(B)における検出可能なシグナルにおける減少は、このプローブがヒトEBER1および2核RNAに特異的にハイブリダイズすることを示している。

10

【図14】

ヒト免疫グロブリン 軽鎖mRNAに対応する標的遺伝子特異的ドメインを保持するプローブ [ 配列番号 : 1 5 ] を用いる 軽鎖ポジティブヒト扁桃組織のISH解析について得られた結果を説明する。

【図15】

ヒト免疫グロブリン 軽鎖mRNAに対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション [ 配列番号 : 2 - 4、配列番号 : 7 - 12、配列番号 : 14, 15 ] を用いるリンパ腫組織のISH解析について得られた結果を説明する。(A)のリンパ腫組織は 軽鎖を過発現し、そして(B)の組織は 軽鎖を過発現する。(B)における検出可能なシグナルの不在は、 軽鎖プローブコレクションが 軽鎖mRNAに特異的であることを示している。

20

【図16】

ヒト免疫グロブリン 軽鎖可変部mRNAに対応する標的遺伝子特異的ドメインを保持するプローブ [ 配列番号 : 19 - 29 ] を用いる 軽鎖ポジティブヒト扁桃組織のISH解析について得られた結果を説明する。

【図17】

ヒト免疫グロブリン 軽鎖mRNAに対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション [ 配列番号 : 19 - 29 ] を用いる 軽鎖ポジティブヒトRPMI 8226細胞系のISH解析について得られた結果を説明する。

【図18】

ヒト免疫グロブリン 軽鎖mRNAに対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション [ 配列番号 : 19 - 29 ] を用いるヒト脾臓組織のISH解析について得られた結果を説明する。(A)の組織は 軽鎖を過発現し、そして(B)の組織は 軽鎖を過発現する。(B)における検出可能なシグナルの不在は、 軽鎖プローブコレクションがヒト 軽鎖mRNAに特異的であることを示している。

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【図19】

CMV即時型RNAに対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション [ 配列番号 : 30 - 32, 配列番号 : 34 - 35, 配列番号 : 38, 配列番号 : 50 ] を用いるサイトメガロウイルス(CMV)ポジティブヒト肺組織のISH解析について得られた結果を説明する。矢印はCMV感染細胞を示す。

40

【図20】

CMV即時型mRNAに対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション [ 配列番号 : 30 - 32, 配列番号 : 34 - 35, 配列番号 : 38, 配列番号 : 50 ] を用いる、CMV即時型RNAの発現がシクロヘキサミドによって誘発されなかったラット9G細胞系のISH解析について得られた結果を説明する。

【図21】

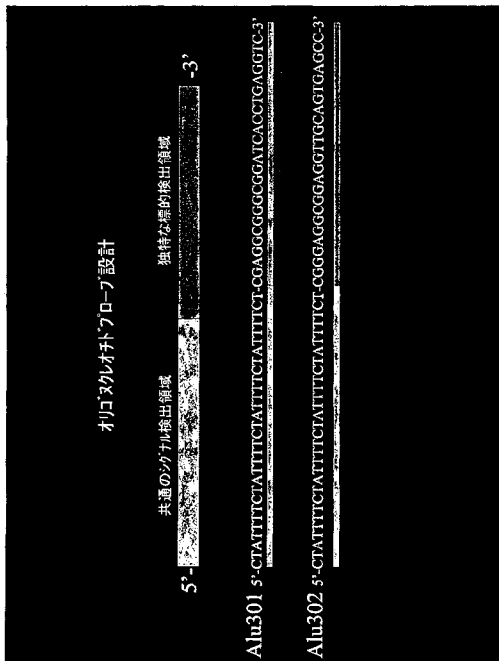
CMV即時型RNAに対応する標的遺伝子特異的ドメインを保持するプローブからなるプローブコレクション [ 配列番号 : 30 - 32, 配列番号 : 34 - 35, 配列番号 : 38, 配列番号 : 50 ] を用いる、CMV即時型RNAの発現がシクロヘキサミドによって誘発されたラット9G細胞系のISH解析について得られた結果を説明する。(A)の組織は

50

倍率 40 X において示され、そして ( B ) の組織は倍率 20 X において示される。

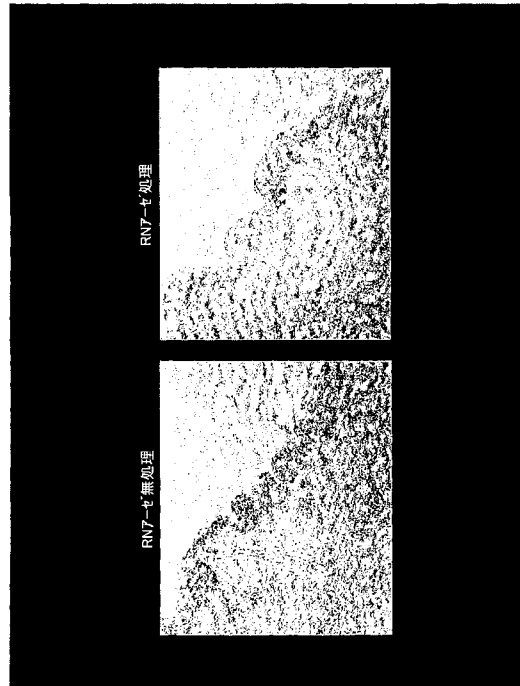
【 図 1 】

1/21



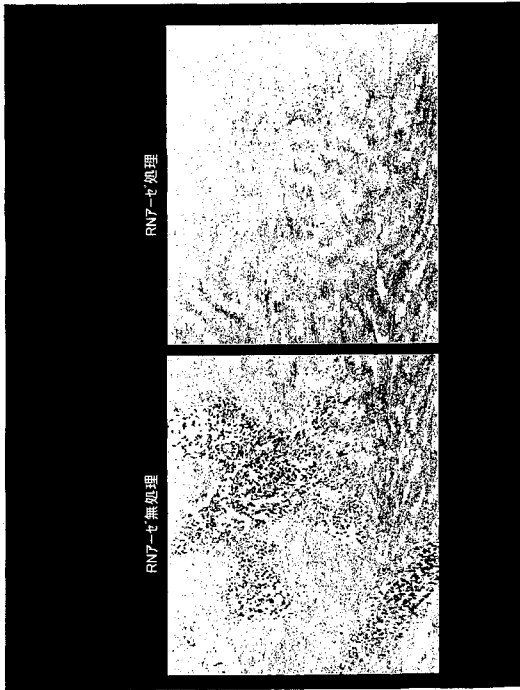
【 図 7 】

7/21



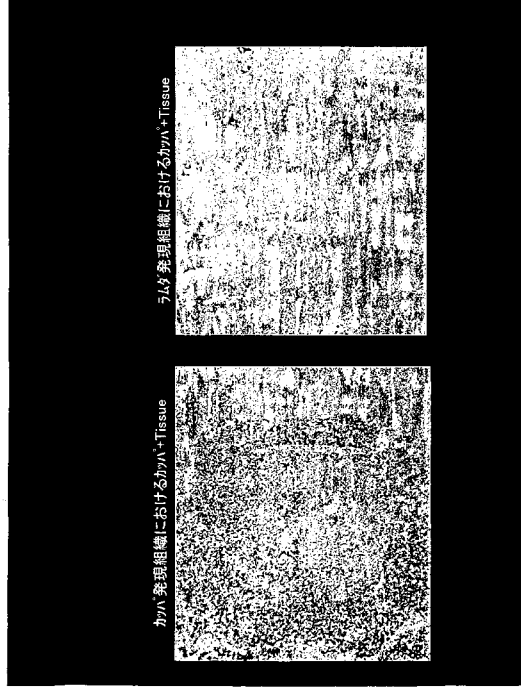
【 図 1 2 】

12/21



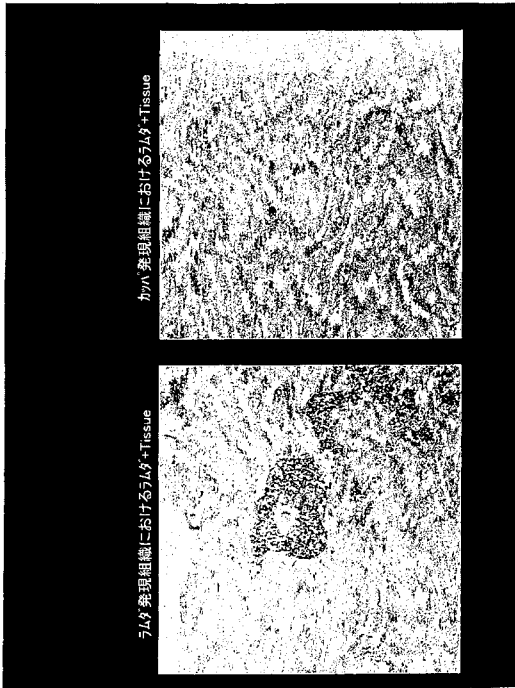
【 図 1 5 】

15/21



【 図 1 8 】

18/21



【国際公開パンフレット】

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
21 March 2002 (21.03.2002)

PCT

(10) International Publication Number  
WO 02/22874 A2

(51) International Patent Classification: C12Q 1/68 Joseph, G. [US/US]; 5357 East 19th Street, Tucson, AZ 85711 (US). CONNAUGHTON, John [US/US]; 9117 Hummaster Road, Laytonsville, MD 20882 (US).

(21) International Application Number: PCT/US01/28014

(22) International Filing Date: 6 September 2001 (06.09.2001) (74) Agent: JONES, Huw, R.; Ventana Medical Systems, Inc., 3865 N. Business Center Drive, Tucson, AZ 85705 (US).

(25) Filing Language: English (81) Designated States (national): AU, CA, JP, MX, US.

(26) Publication Language: English (84) Designated States (regional): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

(30) Priority Data: 60/233,177 15 September 2000 (15.09.2000) US

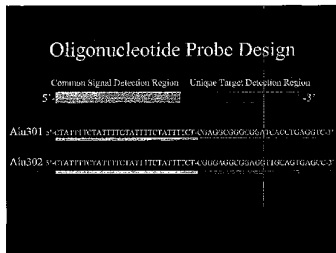
(71) Applicant (for all designated States except US): VENTANA MEDICAL SYSTEMS, INC. [US/US]; 3865 N. Business Center Drive, Tucson, AZ 85705 (US).

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

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

(72) Inventors; and  
(75) Inventors/Applicants (for US only): UTERMOHLEN,

(54) Title: OLIGONUCLEOTIDE SEQUENCE FORMULA FOR LABELING OLIGONUCLEOTIDE PROBE AND PROTEINS FOR IN-SITU ANALYSIS



(57) Abstract: The present invention provides oligonucleotide probes and oligonucleotide probe collections and protein labeling for detecting or localizing a plurality nucleic acid target genes or antigens within a cell or tissue sample. Specifically, the provides collections of oligonucleotide probe for use in *in situ* hybridization analyses in which each probe has a label-domain with the sequence formulas of (CTATTT)<sub>n</sub>, (AAAATAG)<sub>n</sub>, or (TTTATC)<sub>n</sub>, or (GATAAAA)<sub>n</sub>, in which all cases "n" would equal 1 or greater. The present invention provides collections or "cocktails" of oligonucleotide probes for detecting or localizing specific nucleic acid target genes within a cell or tissue sample. The cocktails are useful for detecting the following: the Kappa gene (SEQ ID NOS: 1-16 inclusive); the Lambda gene (SEQ ID NOS: 501-509, 511-513, and 515); the CMV (cytomegalovirus) gene (SEQ ID NOS: 221-241 inclusive); EBEP (Epstein-Barr RNA) gene (SEQ IN NOS: 51-54 inclusive); Alu (SEQ IN NOS: 55-56); PolyA (SEQ ID NO: 57); and the detection tail (SEQ ID NO: 330).



WO 02/22874 A2

WO 02/22874

PCT/US01/28014

**BACKGROUND OF THE INVENTION****1. Field of the Invention**

This invention relates to oligonucleotide probes and collections of oligonucleotide probes for detecting or localizing nucleic acid genes targets within a cell or tissue sample. In particular, the invention relates to collections of oligoprobes.

**2. Background of the Invention**

*In situ* analysis includes *in situ* hybridization and immunohistochemistry. *In situ* hybridization (ISH) employs labeled DNA or RNA probe molecules that are anti-sense to a target gene sequence or transcript to detect or localize targeted nucleic acid target genes within a cell or tissue sample. ISH has proven to be a useful tool in a number of biomedical fields, including developmental biology, cell biology, and molecular biology. ISH has been used, for example, to diagnose genetic disorders, map genes, study gene expression, and localize sites of target gene expression.

Typically, ISH is performed by exposing a cell or tissue sample immobilized on a glass slide to a labeled nucleic acid probe which is capable of specifically hybridizing to a given target gene in the cell or tissue sample (*In Situ Hybridization: Medical Applications* (G. R. Coulton and J. de Bellerche, eds., Kluwer Academic Publishers, 1992); *In Situ Hybridization: In Neurobiology; Advances in Methodology* (J. H. Eberwine, K. L. Valentino, and J. D. Barchas, eds., Oxford University Press, 1994); *In Situ Hybridization: A Practical Approach* (D. G. Wilkinson, ed., Oxford University Press, 1992)). The hybridization of labeled probe molecules to nucleic acids in the cell or tissue sample can then be detected using, for example, radioactive-based direct detection methods, fluorescence-based direct detection methods, or indirect detection methods based on the binding of a fluorescence-labeled protein binding to a hapten such as BrdU, digoxigenin-labeled or biotin-labeled nucleotides incorporated into probes. Hapten-based methods have been further extended to include those molecules to be bonded by binding protein-enzyme conjugates such as antibody-enzyme-conjugates and colorimetric based detection chemistry. In addition, several target genes can be simultaneously analyzed by exposing a cell or tissue sample to a plurality of nucleic acid probes that have been labeled with a plurality of different nucleic

WO 02/22874

PCT/US01/28014

acid tags. For example, a plurality of nucleic acid probes can be labeled with a plurality of fluorescent compounds having different emission wavelengths, thereby permitting simultaneous multicolored analysis to be performed in a single step on a single target cell or tissue sample.

5 A significant problem associated with incorporation of labeled nucleotides into oligonucleotide probes is that the conjugation moieties that are attached to the nucleotide usually interfere with the formation of Watson-Crick base pairing, thus negatively affecting the hybridization of the probe to its target. This has been seen with use of label attached via N4-substituted cytosine nucleotides, because of steric hinderance and the expected shift to the  
10 less reactive state of a secondary amine (as seen with N4 labeled cytosine), as compared to the natural G-C bond formed with an unsubstituted cytosine (a primary amine). Any small change or interference with G-C bonding in a small oligonucleotide (25 to 50 bases) can reduce the ability of these oligos to hybridize with the intended targeted sequence.

There remains a need in the art to develop suitable probes designs for incorporating  
15 labeled nucleotides in oligonucleotide probes. We demonstrate that a few artificial sequences are viable alternatives for probe labeling and also work both singly and in complex oligonucleotide probe mixtures for detecting or localizing nucleic acid target genes within a cell or tissue sample. The development of such generic sequences and labeling strategy for probe collections has wide application in the medical, genetic, and molecular biological arts.

20 This interference due to labeling chemistry and hybridization stringency and kinetics is solved herein by designing the oligo to have at least two distinct functional domains, one domain or sequence to be gene specific and involved in the base pair formation, and the second domain to be an artificial, non-specific sequence (in reference to the sample's genome) comprised of spacing nucleotides and the labeled nucleotide. These elements are  
25 positioned so that these label-nucleotides are more accessible as haptens for binding proteins (immunoglobulin or avidin(s)) and thus do not interfere with Watson-Crick base pairing in the gene-specific domain.

#### SUMMARY OF THE INVENTION

30 The present invention provides a novel strategy to incorporate label into oligonucleotide probes and labeled oligonucleotide probe collections for detecting or

WO 02/22874

PCT/US01/28014

localizing nucleic acid target genes within a cell or tissue sample. In particular, the invention relates to non-gene-specific sequences using sequence formulas for making repetitive polymers of such sequences which can be incorporated into collections of oligonucleotide probes for use in *in situ* hybridization analyses. In addition, using labeled synthetic

5 oligonucleotide polymers, based on sequence formulas, when conjugated to binding proteins, i.e. immunoglobulins, is a very effective and controlled process for labeling such proteins used in immunohistochemical analysis. The present invention provides collections or "cocktails" of oligonucleotide probes for detecting or localizing specific nucleic acid target genes within a cell or tissue sample. The cocktails are useful for detecting the following: the

10 Kappa gene (SEQ ID NOS: 1-16 inclusive); the Lamba gene (SEQ ID NOS: 501-509, 511-513, and 515); the CMV (cytomegalovirus) gene (SEQ ID NOS: 221-241 inclusive); EBER (Epstein-Barr early RNA) gene (SEQ ID NOS: 51-54 inclusive); Alu (SEQ ID NOS: 55-56); PolyA (SEQ ID NO: 57); and the detection tail (SEQ ID NO:330).

The invention is directed to an oligonucleotide label-domain comprising the sequence

15 (CTATTTT)<sub>n</sub> and its complement (AAAATAG)<sub>n</sub> wherein "n" is at least 1.

The invention is also directed to an oligonucleotide probe having at least two distinct functional domains, a first domain comprising the label-domain of claim 2, and a second domain comprising a gene-specific target sequence.

The invention is also directed to a probeset for detecting Kappa immunoglobulin light

20 chain mRNA or corresponding heteronuclear RNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 401 through 416, inclusive.

The invention is also directed to a probeset for detecting Lambda immunoglobulin light chain mRNA or corresponding heteronuclear RNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 501 through 509, 511-513, and 515.

25 The invention is also directed to A probeset for detecting cytomegalovirus (CMV) immediate early RNA and /or corresponding mRNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 221 through 241

WO 02/22874

PCT/US01/28014

The invention is also directed to a probeset for detecting Epstein Barr virus (EBV) early RNA, RNA 1 and RNA 2, (EBER) wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 51 through 54.

The invention is also directed to a probeset for detecting Human Alu repetitive satellite genomic DNA sequences wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 301 and 302.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

#### 10 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a generic probe structure of the two-domain probe design. This is the oligonucleotide design used for the probes in the gene specific cocktails described in the following examples. Each probe is composed of two domains: a 5' labeling domain and a 3' target gene target gene-specific domain. The labeling domain consists of this specific sequence (CTATTTT)<sub>n</sub>, wherein each cytosine may be labeled with a fluorophore or a cytosine-hapten conjugate, the hapten being fluorescein in this embodiment. This illustration specifically shows nucleic acid sequences for the 301 (SEQ ID NO: 55) and 302 (SEQ ID NO: 56) probes, each of which possesses target gene gene-specific domains corresponding to human repetitive Alu sequences and labeling domains having a fluorescein hapten.

Figure 2 illustrates the results obtained for *in situ* hybridization (ISH) analysis of human skin tissue using a probe comprising the labeling domain (330 probe; SEQ ID NO: 58). The absence of a detectable signal indicates that the sequence formula, (CTATTTT)<sub>n</sub>, of the labeling domain common to the oligonucleotides used in these ISH examples is non-specific, and non-reactive in its ability to form Watson-Crick base pairing with human nucleic acid sequences because it does not hybridize.

Figure 3 illustrates the results obtained for ISH analysis of human skin tissue using a probe comprising the labeling domain and a poly d(T) target gene-specific domain (320 probe; SEQ ID NO: 57). The presence of a detectable signal localized to the cytoplasm

WO 02/22874

PCT/US01/28014

indicates that this probe is capable of specifically hybridizing to polyadenylated region of messenger RNA.

Figures 4A-4B illustrate the results obtained for ISH analysis of human skin tissue using the 320 probe, wherein the tissue sample was not treated with ribonuclease A prior to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The decrease in detectable signal in (B) indicates that this probe specifically hybridizes to polyadenylated region common to messenger RNA.

Figures 5A-5B illustrate the results obtained for ISH analysis of human spleen tissue using the 320 probe, wherein the hybridization and stringency wash were performed at room temperature (A), or at 37°C (B). This result illustrates that the intensity of color is related to the stringency of hybridization conditions, with the more intense color indicating less stringent conditions.

Figure 6 illustrates the results obtained for ISH analysis of the human Raji cell line using the 320 probe. This shows that this probe design also is functional with embedded cell lines as well as embedded tissue.

Figure 7 illustrates the results obtained for ISH analysis of the human Raji cell line using a probe collection consisting of the 301 and 302 probes.

Figure 8 illustrates the results obtained for ISH analysis of the human HT cell line using a probe collection consisting of the 301 and 302 probes.

Figure 9 illustrates the results obtained for ISH analysis of a rat cell line using a probe collection consisting of the 301 and 302 probes. The absence of a detectable signal indicates that this probe collection is specific for human nucleic acid sequences.

Figure 10 illustrates the results obtained for ISH analysis of an Epstein-Barr virus (EBV)-negative human HT cell line using a probe possessing a target gene-specific domain corresponding to EBV EBER nuclear RNA [SEQ ID NO: 51 through SEQ ID NO: 54].

Figure 11 illustrates the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54].

Figure 12 illustrates the results obtained for ISH analysis of human tonsil tissue using a probe collection consisting of probes possessing target gene-specific domains

WO 02/22874

PCT/US01/28014

corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54].

5 Figures 13A-13B illustrate the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54], wherein the tissue sample was not treated with ribonuclease A prior to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The decrease in detectable signal in (B) indicates that this probe specifically hybridizes to human EBER 1 and EBER 2 nuclear RNA.

10 Figure 14 illustrates the results obtained for ISH analysis of kappa light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NO:15].

15 Figure 15 illustrates the results obtained for ISH analysis of lymphoma tissues using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin kappa light chain mRNA [SEQ ID NOS: 2-4, SEQ ID NOS:7-12, SEQ ID NOS: 14, 15]. The lymphoma tissue in (A) over expresses the kappa light chain and the tissue in (B) over expresses the lambda light chain. The absence of a detectable signal in (B) indicates that the kappa light chain probe collection is specific to kappa light chain mRNA.

20 Figure 16 illustrates the results obtained for ISH analysis of lambda light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain variable region mRNA [SEQ ID NOS:19 through 29].

25 Figure 17 illustrates the results obtained for ISH analysis of a lambda light chain-positive human RPMI 8226 cell line using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NOS:19 through 29].

30 Figures 18A-18B illustrate the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NOS:19 through 29]. The tissue in (A) over expresses the lambda light chain and the tissue in (B)

WO 02/22874

PCT/US01/28014

over expresses the kappa light chain. The absence of a detectable signal in (B) indicates that the lambda light chain probe collection is specific to human lambda light chain mRNA.

Figure 19 illustrates the results obtained for ISH analysis of cytomegalovirus (CMV)-positive human lung tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA [SEQ ID NOS:30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50]. [CMV infected cell]

Figure 20 illustrates the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has not been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early mRNA [SEQ ID NOS:30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50].

Figures 21A-21B illustrate the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA [SEQ ID NOS:30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50]. The tissue in (A) is shown at a magnification of 40X and the tissue in (B) is shown at a magnification of 20X.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides oligonucleotide probes and oligonucleotide probe collections for detecting or localizing nucleic acid target genes within a cell or tissue sample. In particular, the invention relates to collections of oligonucleotide probes for use in *in situ* hybridization analyses.

More specifically, this invention relates to the use of specific sequence formulas for nucleotide polymers or label-domains to attach a detectable moiety ( a label) to oligonucleotide probes or proteins. The specific utility of these sequences or derivatives thereof, is the inert or non-reactive characteristic that does not hybridize to human DNA or RNA at a detectable level under standard stringency of hybridization conditions. These label-domains or polymers were demonstrated to be useful generic sequences for incorporation into oligonucleotide probes for detecting gene-specific sequences within cells or tissue samples in *in situ* hybridization analyses. Additionally, this inert set of sequences

WO 02/22874

PCT/US01/28014

are useful for attaching a label to immunoglobulins or other proteins for detecting haptens and antigens in immunohistochemical analyses.

As used herein, the terms "probe" or "oligonucleotide probe" refers to a nucleic acid molecule used to detect a complementary nucleic acid target gene.

5 As used herein, the term "hybridization" refers to the process whereby complementary nucleic acid sequences join to form a double-stranded nucleic acid molecule. By labeling the target nucleic acid molecule with, for example, a radioactive or fluorescent tag, interactions between probe and target genes can be detected.

10 The oligonucleotide probes and oligonucleotide probes of the collections of the present invention are synthesized using conventional methods. See e.g., *Methods in Molecular Biology, Vol 20: Protocols for Oligonucleotides and Analogs* 165-89 (S. Agrawal, ed., 1993); *Oligonucleotides and Analogues: A Practical Approach* 87-108 (F. Eckstein, ed., 1991).

15 In a preferred embodiment of the present invention, oligonucleotide probes possess two distinct domains: a 5' (or labeling) domain and a 3' (or gene-specific target) domain (See Figure 1A). In more preferred embodiments of the present invention, the oligonucleotide probe possesses a labeling domain which consists of the sequence (CTATTT)<sub>n</sub>. Other embodiments are also demonstrated herein, including a triple-domain  
20 embodiment having two terminal labeling domains, and a central gene-specific target domain. Specifically, SEQ ID NOS: 125-126 depict this labeling scheme. Yet a further preferred embodiment of a labeling domain is TC(TTTTATC)<sub>n</sub> or its complement. This sequence is predicted to be as unique as the (CTATTT)<sub>n</sub> label-domain. The oligonucleotide probes of the present invention are labeled so that hybridization between said  
25 probes and target nucleic acids in a particular cell or tissue can be detected. Labels that are acceptable for use in *in situ* hybridization (ISH) analysis are known to those with skill in the art. Such labels permit interactions between probe and target genes to be detected using, for example, radioactive-based direct detection methods, fluorescence-based direct detection methods, digoxigenin-labeled or biotin-labeled probes coupled with fluorescence-based  
30 detection methods, or digoxigenin-labeled or biotin-labeled probes coupled with antibody-enzyme-based detection methods. In preferred embodiments of the present invention, oligonucleotide probes are labeled with fluorescein. In more preferred embodiments of the

WO 02/22874

PCT/US01/28014

present invention, the oligonucleotide probe possesses a labeling domain which consists of the sequence (CTATTTT)<sub>n</sub>, wherein the cytosine nucleotides may be labeled with a fluorophore for direct detection, or a hapten for indirect detection. In either, the fluorescein-cytosine nucleotide conjugate and the fluorescein molecule is linked at the N4 position of cytosine through an OBBA linkage (See Mishra *et al.*, U.S. Patent No. 5,684,142, which is incorporated herein by reference). In a preferred embodiment, the density of fluorophore attached to the label-domain is at least 7 mole percent, preferably at least 10 mole percent, and most preferably at least 16 mole percent, when measured against the label-domain solely. For example, if probe 401 is considered (a 2-domain probe) it comprises a label-domain of 30 bases including a 3' terminal CT wherein the C is also labeled, the mole percent is 5/30 = 16.7 mole percent label. In the overall probe, the mole percent is 8.3.

In some embodiments of the present invention, several target genes are simultaneously analyzed by exposing a cell or tissue sample to a plurality of nucleic acid probes that have been labeled with a plurality of different nucleic acid tags. For example, a plurality of nucleic acid probes can be labeled with a plurality of fluorescent compounds having different emission wavelengths, thereby permitting simultaneous multicolored analysis to be performed in a single step on a single target cell or tissue sample.

The oligonucleotide probes and oligonucleotide probe collections of the present invention may be used in ISH analysis to detect or localize nucleic acid target genes within a cell or tissue sample. ISH may be performed as described, for example, in *In Situ Hybridization: Medical Applications* (G. R. Coulton and J. de Belleruche, eds., Kluwer Academic Publishers, 1992); *In Situ Hybridization: In Neurobiology: Advances in Methodology* (J. H. Eberwine, K. L. Valentino, and J. D. Barchas, eds., Oxford University Press, 1994); or *In Situ Hybridization: A Practical Approach* (D. G. Wilkinson, ed., Oxford University Press, 1992).

The preferred embodiment of the probes and probe collections of the present invention are best understood by referring to Figures 1-21 and Examples 1-2. The Examples, which follow, are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

WO 02/22874

PCT/US01/28014

**EXAMPLE 1****Probe Collection Preparation**

Probe collections consisting of a plurality of oligonucleotide probes of 55 to 60 bases in length were designed as follows. In this Example, each oligonucleotide probe possessed two distinct domains: a 5' (or labeling) domain and a 3' (or target gene-specific) domain (See Figure 1).

In this embodiment, the labeling domain consists of the sequence (CTATTTT)<sub>n</sub>, wherein the cytosine nucleotide represents a fluorescein-cytosine nucleotide conjugate and the fluorescein molecule is linked at the N4 position of cytosine through an OBEB linkage.

The target gene-specific domain consists of a 25-30 base sequence that is complementary to a specific nucleic acid target gene. Oligonucleotide probes were designed to possess target gene-specific domains corresponding to the human immunoglobulin kappa light chain variable region (See Table 1; oligonucleotide probes 401-416), the human immunoglobulin lambda light chain variable region (oligonucleotide probes 501-515), human cytomegalovirus (CMV) sequences (oligonucleotide probes 221-241), human Epstein-Barr virus (EBV) EBER (Epstein-Barr early RNA) sequences (oligonucleotide probes 100A2, 100C2, 100A1, and 100B1), human repetitive Alu sequences (oligonucleotide probes 301 and 302), and poly d(T) (oligonucleotide probe 320).

**EXAMPLE 2****Label-domain design: Alu repetitive sequence probe**

Four probes all against the Alu human repetitive sequence were used to evaluate label-domain design. The probes numbered 301 (SEQ ID NO: 55), 301A (SEQ ID NO:116), 301A2/2 (SEQ ID NO: 121), and 301A3/2 (SEQ ID NO: 122) are shown in Table 1.

The four probes were evaluated at the concentrations of 100, 75, 50, and 25 ng/ml per mL of probe in the reaction, respectively. This hybridization analysis was done manually, using standard protocols. The target, paraffin-embedded cell line MBA MD 468 (Oncor INFORM™ Her-2/neu Control Slides, Cat. No. S8100, Level 1, available from Ventana Medical Systems, Inc., Tucson, AZ) was the target sample and was processed by removing paraffin by standard xylene methods. The tissue was subjected to Ventana's Protease 1 for 12 minutes at 50 degrees C as a 1:2 dilution with Ventana's APK buffer. The hybridization reaction was accomplished with the addition of probe diluent as 100 ul probe (25%

WO 02/22874

PCT/US01/28014

formamide, 5% dextran sulfate, 2X SSC, 1% Triton) to a residual 100 ul volume of 2XSSC/Triton X-100. The slide was heated to 85 degrees C for 5 minutes and then incubated for 1 hr at 37 degrees C. Standard SSC washes followed for removing excess probe. The hybrids were detected with an antibody against FITC. The mouse antibody was detected  
5 colormetrically using Ventana Enhanced Alkaline Phosphatase Blue Detection (cat# 760-061). Unless otherwise indicated, all reagents were obtained from Ventana Medical Systems, Inc., Tucson, AZ. The results were observed by colormetric detection using brightfield microscopy.

The results of these experiments were that signal intensity was a function of the total  
10 number of fluorescein hapten conjugated to the probe and signal was of the specific label-domain design. The greater the number of fluoresceins per probe molecule, the greater the signal observed. Comparison of design and placement of haptens on the probe showed that this was not a factor in signal intensity. The two probes that contained five fluoresceins, (301A3/2 (SEQ ID NO: 122) and 301 (SEQ ID NO:55) both yielded equivalent signal. These  
15 two probes yielded greater signal than seen for 301A2/2, a probe with a split label-domain design with four fluoresceins. The probe 301A2/2 yielded a signal greater than probe 301A a probe with a single label-domain design at the 5' end and with three fluoresceins.

### EXAMPLE 3

#### 20 Label Domain Design: EBER probes

This experiment compared two label-domain designs and sequences to determine whether greater spacing between the fluorescein haptens improves the production of signal during probe detection steps during *in situ* hybridization analysis.

The tissue used was an EBV-infected human spleen tissue fixed in neutral buffered  
25 formalin paraffin embedded section of 4-micron thickness placed on silane plus glass microscope slides. The tissue sections were deparaffinized on a Ventana DISCOVERY™ machine, followed by a 6-min digestion with Ventana's Protease 1, at a temperature of 37 C. The probe was dissolved in hybridization buffer diluent at a concentration of 50 ng/mL as a 100 ul applied to an equal volume of 2 X SSC/ Triton X-100 residual volume left on the slide  
30 after prepared by the Ventana Medical Systems, Inc. automated ISH staining system, Discovery. The probe diluent-mixed with the residual volume on slide for 6 min at 37 C,

WO 02/22874

PCT/US01/28014

then the solution was heated to 85C and held there for a total of 10 min. The slide was then taken to a 37C temperature and held at that temperature for 1 hour. All of these aqueous reactions on the slide were all done under a film of LIQUID COVERSLIP™, to prevent evaporative loss of water during processing. Each slide after hybridization was washed 3 times with 2X SSC/Triton solution, with a 6 min incubation between each wash, the slide volume being approximately 300 ul (+/- 10% vol). The hybrids were detected with an antibody against FITC. The mouse antibody was detected colorimetrically using Ventana Enhanced Alkaline Phosphatase Blue Detection (cat# 760-061).

The two oligonucleotide probes used for this study probe 100A1 (SEQ ID NO: 53) and 1002A32 (SEQ ID NO: 120). The two differences between these probes were the label-domain sequence and structure. The probe 100A1 label domain was 5' to gene target domain, contained 5 fluoresceins attached to cytosine residues via the OBEA linker, with the sequence formula of (CTATTTT)<sub>4</sub>CT (SEQ ID NO: 58). The label domain of the oligo probe 1002A32, was similar, (SEQ ID NO:125). Besides the different sequence the primary difference was that the fluoresceine labeled cytosines were spaced 10 bases apart compared to the oligo 100A1 the cytosine spacing was closer at 7 bases apart. The result of this comparison as deduced by H score analysis were that these oligonucleotide were equivalent as to the amount of signal generated on the slide. The data was that for 100A2, for the 368 cells analysed in a total of 3 fields the H score was 106, and for probe 1002A32 for the 345 cell analysed in three field the H score was 109. The H score is a spectrographic analysis done with microscope that factors into the score background to signal ratio on the tissue section to yield a relative comparison of total target specific signal on the slide. (See reference Giroud, F. Perrin C, and Simony Lafontaine, J.; Quantitative Immunocytochemistry and Immunohistochemistry. Third Conference of the European Society for Analytical Cellular Pathology, 1994; and AutoCyte Quic Immuno User's Manual, 1998, document number PA-029, Co AutoCyte Inc. Burlington NC 2721). The histograms and the score sheet indicated that each oligo were equally efficient in yielding a colorimetric signal. This indicates that the position of the label domain can be either 3 prime or 5 prime to the gene target sequence or the gene target sequence can be positioned between two label domains.

#### EXAMPLE 4

##### In Situ Hybridization

WO 02/22874

PCT/US01/28014

The probe collections prepared in Example 1 were first diluted in a solution consisting of 20% dextran sulfate (wt/vol), 50% formamide (vol/vol), 2X SSC, 10 mM Tris-HCl, 5 mM EDTA, and 0.05% Brij-35, at a final pH of 7.3. Probe collections were then mixed with an equal volume of a solution consisting of 2X SSC and 0.05% Triton X-100.

5 Samples for ISH analysis were prepared by cutting formalin-fixed and paraffin-embedded cells or tissue samples into 4  $\mu$ m sections and placing the sections onto a glass slide. Subsequent processing and ISH of samples was carried out in an automated device, such as the DISCOVERY™ Automated ISH/IHC Stainer (Ventana Medical Systems, Inc., Tucson, AZ) described in co-owned and co-pending U.S. Patent App. Serial Nos. 60/076,198  
10 and 09/259,240, both incorporated herein by reference. To remove paraffin from the samples, the slides were immersed in an aqueous solution, heated for approximately 20 minutes, and then rinsed. The automated deparaffinization procedure is more fully described in U.S. Serial No. 60/099,018, 09/259,240 both incorporated herein by reference. The samples were then treated with protease and the slides were heated to 85°C (for hybridization  
15 to RNA target genes) or 90-95°C (for hybridization to DNA target genes) for 4 to 10 minutes.

Hybridization reactions were typically performed in a hybridization buffer consisting of 10% dextran sulfate (wt/vol), 25% formamide (vol/vol), 2X SSC, 5 mM Tris, 2.5 mM EDTA, 0.025% Brij-35, 0.25% Triton X-100, and between 25 to 125 ng/mL of each individual probe molecule. ISH reactions were performed at between 37°C to 54°C. For ISH  
20 using the probe collections described in Example 1, hybridization reactions were optimally carried out for 1 hr at 47°C (except for the poly d(T) probe, wherein the hybridization reaction was optimally carried out at 37°C for 1 hr).

The hybridization of fluorescein-labeled probe molecules to a particular target gene in the sample was detected by using a sequential series of binding proteins, i.e., secondary  
25 antibody detection. However, it is equally possible to use detect detection when visualizing the bound probes. In secondary detection, first, an anti-fluorescein mouse monoclonal antibody directed against the fluorescein-labeled probe molecule was added to the sample. Next, a biotin-labeled polyclonal goat antibody directed against the mouse antibody was added to the sample. Finally, hybridization reactions were colorimetrically detected using a 5-  
30 bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate. This technique, termed "secondary antibody detection," is routine for one of skill in the art.

WO 02/22874

PCT/US01/28014

Primary and secondary antibodies are available from numerous suppliers, including Ventana Medical Systems, Tucson, AZ, which are optimized for use on the Ventana autostaining systems (BS<sup>®</sup>, NexES<sup>®</sup>, DISCOVERY<sup>™</sup>, and BENCHMARK<sup>™</sup>).

5 Figures 2-21 illustrate the results obtained for *in situ* hybridization analysis of various cell lines or tissue samples using the probes disclosed and claimed herein having the structural motif illustrated in Figure 1 or probe collections consisting of such probes.

Figure 1 illustrates a generic probe structure of the two-domain probe design. This is the oligonucleotide design used for the probes in the gene specific cocktails described in the following examples. Each probe is composed of two domains: a 5' labeling domain and a 3' target gene target gene-specific domain. The labeling domain consists of this specific sequence (CTATTTT)<sub>n</sub>, wherein the cytosine nucleotide is a cytosine-hapten conjugate, the hapten being fluorescein in this embodiment. This illustration specifically shows nucleic acid sequences for the 301 (SEQ ID NO: 55) and 302 (SEQ ID NO: 56) probes, each of which possesses target gene gene-specific domains corresponding to human repetitive Alu sequences and labeling domains having a fluorescein hapten.

15 Figure 2 illustrates the results obtained for *in situ* hybridization (ISH) analysis of human skin tissue using a probe comprising the labeling domain (330 probe; SEQ ID NO: 58). The absence of a detectable signal indicates that the sequence formula, (CTATTTT)<sub>n</sub>, of the labeling domain common to the oligonucleotides used in these ISH examples is non-specific, and non-reactive in its ability to form Watson-Crick base pairing with human nucleic acid sequences because it does not hybridize.

20 Figure 3 illustrates the results obtained for ISH analysis of human skin tissue using a probe comprising the labeling domain and a poly d(T) target gene-specific domain (320 probe; SEQ ID NO: 57). The presence of a detectable signal localized to the cytoplasm indicates that this probe is capable of specifically hybridizing to polyadenylated region of messenger RNA.

25 Figures 4A-4B illustrate the results obtained for ISH analysis of human skin tissue using the 320 probe, wherein the tissue sample was not treated with ribonuclease A prior to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The decrease in detectable signal in (B) indicates that this probe specifically hybridizes to polyadenylated region common to messenger RNA.

WO 02/22874

PCT/US01/28014

Figures 5A-5B illustrate the results obtained for ISH analysis of human spleen tissue using the 320 probe, wherein the hybridization and stringency wash were performed at room temperature (A), or at 37°C (B). This result illustrates that the intensity of color is related to the stringency of hybridization conditions, with the more intense color indicating less stringent conditions.

Figure 6 illustrates the results obtained for ISH analysis of the human Raji cell line using the 320 probe. This shows that this probe design also is functional with embedded cell lines as well as embedded tissue.

Figure 7 illustrates the results obtained for ISH analysis of the human Raji cell line using a probe collection consisting of the 301 and 302 probes.

Figure 8 illustrates the results obtained for ISH analysis of the human HT cell line using a probe collection consisting of the 301 and 302 probes.

Figure 9 illustrates the results obtained for ISH analysis of a rat cell line using a probe collection consisting of the 301 and 302 probes. The absence of a detectable signal indicates that this probe collection is specific for human nucleic acid sequences.

Figure 10 illustrates the results obtained for ISH analysis of an Epstein-Barr virus (EBV)-negative human HT cell line using a probe possessing a target gene-specific domain corresponding to EBV EBER nuclear RNA [SEQ ID NO: 51 through SEQ ID NO: 54].

Figure 11 illustrates the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54].

Figure 12 illustrates the results obtained for ISH analysis of human tonsil tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54].

Figures 13A-13B illustrate the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54], wherein the tissue sample was not treated with ribonuclease A prior to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The

WO 02/22874

PCT/US01/28014

decrease in detectable signal in (B) indicates that this probe specifically hybridizes to human EBER 1 and EBER 2 nuclear RNA .

Figure 14 illustrates the results obtained for ISH analysis of kappa light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NO:15].

Figure 15 illustrates the results obtained for ISH analysis of lymphoma tissues using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin kappa light chain mRNA [SEQ ID NOS: 2 - 4, SEQ ID NOS:7-12, SEQ ID NOS: 14, 15]. The lymphoma tissue in (A) over expresses the kappa light chain and the tissue in (B) over expresses the lambda light chain. The absence of a detectable signal in (B) indicates that the kappa light chain probe collection is specific to kappa light chain mRNA.

Figure 16 illustrates the results obtained for ISH analysis of lambda light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain variable region mRNA [SEQ ID NOS:19 through 29].

Figure 17 illustrates the results obtained for ISH analysis of a lambda light chain-positive human RPMI 8226 cell line using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NOS:19 through 29].

Figures 18A-18B illustrate the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NOS:19 through 29]. The tissue in (A) over expresses the lambda light chain and the tissue in (B) over expresses the kappa light chain. The absence of a detectable signal in (B) indicates that the lambda light chain probe collection is specific to human lambda light chain mRNA.

Figure 19 illustrates the results obtained for ISH analysis of cytomegalovirus (CMV)-positive human lung tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA [SEQ ID NOS:30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50]. Arrow indicates CMV infected cell.

WO 02/22874

PCT/US01/28014

Figure 20 illustrates the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has not been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA [SEQ ID NOS:30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50].

Figures 21A-21B illustrate the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA [SEQ ID NOS:30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50] expression of the CMV immediate early RNA with cyclohexamide. The tissue in (A) is shown at a magnification of 40X and the tissue in (B) is shown at a magnification of 20X.

Table 1

Proba ID	Sequence	SEQ ID
401	5'-CTATTTCTATTTCTATTTCTATTTCT CCAGAGTAGCAGGAGCCCGCAGCTGAGC-3'	1
402	5'-CTATTTCTATTTCTATTTCTATTTCT GGAATGGAGACTGGGTCACTGGATGTCACA-3'	2
403	5'-CTATTTCTATTTCTATTTCTATTTCT GCAAGCGATGGTGAATCTGTCTCTACAGC-3'	3
404	5'-CTATTTCTATTTCTATTTCTATTTCT TCTGTCCAGATCCACTGSCACTGAACTT-3'	4
405	5'-CTATTTCTATTTCTATTTCTATTTCT GCAGCCACAGTTCGCTTCATCTGCACTTC-3'	5
406	5'-CTATTTCTATTTCTATTTCTATTTCT TTTCBACTGCTCATCAGATGGCGGAAAGT-3'	6
407	5'-CTATTTCTATTTCTATTTCTATTTCT AAGTTAATCAGCAGGCACACACAGAGSCA-3'	7
408	5'-CTATTTCTATTTCTATTTCTATTTCT GCGTTATCCACCTTCCACATGACTTGGC-3'	8
409	5'-CTATTTCTATTTCTATTTCTATTTCT TAGGTCCTGCTCCTGCTGCTGCTGCTG-3'	9
410	5'-CTATTTCTATTTCTATTTCTATTTCT GTAGTCTGCTTTGCTCAGGCTCAGGTTGCT-3'	10
411	5'-CTATTTCTATTTCTATTTCTATTTCT GATGGTACTTCCAGGCTGAGACTTGT-3'	11
412	5'-CTATTTCTATTTCTATTTCTATTTCT CTCTCCCTGTGAGAGCTCTTTGTGACGG-3'	12
413	5'-CTATTTCTATTTCTATTTCTATTTCT TGGAACTGAGGAGCAGTGGGGCACTTC-3'	13
414	5'-CTATTTCTATTTCTATTTCTATTTCT GAAAAAGGATCAGAGCCCAAGGATGGAG-3'	14
415	5'-CTATTTCTATTTCTATTTCTATTTCT AGATGACTGGAGGACCGCAATAGGGTAG-3'	15

WO 02/22874

PCT/US01/28014

416	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	GCATATTAAGCCAGGAGGAGGAGGGGG-3'	16
501	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	CCTGAGTGAGGAGGGTGGAGGACAGCAGG-3'	17
502	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	AGACCCAGACACGGAGGCGAGGCTGATCAG-3'	18
503	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	TGTTGGTCCAGTGCAGGAGATGATGATCG-3'	19
504	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	TAAATCATGATTTTGGGGCTTTGCTGGG-3'	20
505	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	TGTGCCAGACTTGGAGCCAGAGAGCGAT-3'	21
506	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	ATCATCAGCCCTGCTCCAGCCGGAGCC-3'	22
507	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	GGTCCCCTGGGCGAAGCCACAGTGAAC-3'	23
508	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	TTATGAGCACACCAGTGTGGCTTGTGG-3'	24
509	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	CTGTCAGGCGTCAAGCTCAGATAGCTGCT-3'	25
511	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	ATGCGTACCTGCGAGCTGTAGCTTCTGTG-3'	26
512	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	ATTCGTGAGGGCCACTGCTCTCCAGG-3'	27
513	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	CCTCCCTGGGATCCAGCAGCTCAGTCTC-3'	28
515	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	TGAGGTTTATGTGTCCAGGGAGAGGGC-3'	29
221	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	GGAGGTCAAACAGCGTGGATGGCG-3'	30
222	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	GAGGCTGATCGGTCCCGGTGCTT-3'	31
223	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	AATCGCGTCCAAAGCACCGTTCC-3'	32
224	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	TAAAACCTGCGGCACTGGGGACGG-3'	33
225	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	ACCCGAGATTGCGGTGAGATCCCA-3'	34
226	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	GAGCAAGGAGCTGCCGAGGACCAT-3'	35
227	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	ACACGGTGGTGGTGGCAATCGTGC-3'	36
228	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	TCCAAATGCGTCAAGCGGTCAAGC-3'	37
229	5'-CTATTTCTATTTTCTATTTTCTATTTTCT	AGCTBCCTGCATCTCTCTGCGC-3'	38

WO 02/22874

PCT/US01/28014

238	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT	TCTCAGAGGATCGGCCCCCGAATG-3'	47
239	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT	CCTCATCTGACTCCTCGGCGATGGC-3'	48
240	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT	CGGGTACAGGGGATCTGCGGGTGA-3'	49
241	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT	GGTGGGGTCTCTGCTCCAGAGG-3'	50
100A2	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT	GACTCGGGTCTGGTACCCGCACT-3'	51
100C2	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT	GGAGGCTCTCTCTCCCTCCCGG-3'	52
100A1	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT	CCACAGACCCGCTCCACCCCGG-3'	53
100B1	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT	GGCTACAGCCACACACGACTCC-3'	54
301	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT	CGAGCCGGCGGATCACCAGGTC-3'	55
302	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT	CGGAGGCGGAGGTGCGATGAGCC-3'	56
320	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT	TTTTTTTTTTTTTTTTTTTTTTTTTT-3'	57
301A	5'-CTATTTTCTATTTTCTTTTT	CGAGCCGGCGGATCACCAGGTC-3'	116
302C	5'-CTATTTTCTATTTTCTTTTT	CGGAGGCGGAGGTGCGATGAGCC-3'	117
302A4	5'-CTATTTTACTTTTATATTTTCTATTTTCT	CGGAGGCGGAGGTGCGATGAGCC-3'	118
302A3/2	5'-CTATTTTACTTTTATATTTTCT	CGGAGGCGGAGGTGCGATGAGCC ACTATTTTACTT-3	119
1002A32	5'-CTATTTTACTTTTATATTTTCT	GGCTGCGGTGGTACACCCGAC TACTATTTTACTT-3'	120
301A2/2	5'-CTATTTTCTTT	CGAGCCGGCGGATCACCAGGTC TTTCTTTTACTT-3	121
301A3/2	5'-CTATTTTACTTTTATATTTTCT	CGAGCCGGCGGATCACCAGGTC ACTATTTTACTT-3'	122

Table 2

Probe ID	Sequence	SEQ ID
	5'-CTATTTTCTATTTTCTTTTT	123
	5'-CTATTTTACTTTTATATTTTCTATTTTCT	124
330	5'-CTATTTTCTATTTTCTATTTTCTATTTTCT	58
	5'-CTATTTTACTTTTATATTTTCT.....ACTATTTTACTT-3	125
	5'-CTATTTTCTTT.....TTCTTTTACTTT-3	126

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

WO 02/22874

PCT/US01/28014

## WE CLAIM:

1. An oligonucleotide label-domain comprising the sequence (CTATTT)<sub>n</sub> and its complement (AAAATAG)<sub>n</sub> wherein "n" is at least 1.  
5
2. The oligonucleotide label-domain of claim 1 detectably labeled with a reporter molecule, or a hapten molecule.
3. The oligonucleotide label-domain of claim 2 wherein the hapten is fluorescein linked to the N4 nitrogen of cytosine through an OB EA linker.  
10
4. The oligonucleotide label-domain of claim 1 wherein the reporter molecule is a fluorophore.
- 15 5. The oligonucleotide label-domain of claim 1 wherein the fluorophore is present at a density of greater than 7 mole percent.
6. The oligonucleotide label-domain of claim 1 wherein the label-domain has the sequence TC(TTTTATC)<sub>n</sub> ( or its complementary formula).  
20
7. The oligonucleotide label-domain of claim 1 wherein the sequence is SEQ ID NO: 58.
8. The oligonucleotide label-domain of claim 2 wherein at least 7 mole percent of the cytosines are linked to a detectable moiety by an OB EA linker.

WO 02/22874

PCT/US01/28014

9. An oligonucleotide probe having at least two distinct functional domains, a first domain comprising the label-domain of claim 2, and a second domain comprising a gene-specific target sequence.
- 5
10. The oligonucleotide probe of claim 9 wherein the label-domain is located at the 5' end of the oligonucleotide probe, and the gene-specific target sequence being 3' to the label-domain.
- 10
11. The oligonucleotide probe of claim 9 wherein the label-domain is located at the 3' end of the oligonucleotide probe, and the gene-specific target sequence is 5' to the label-domain.
12. An oligonucleotide probe having three distinct functional domains, a first domain comprising the label-domain of claim 2, a second domain comprising a gene-specific target sequence, and a third domain comprising another label-domain, wherein said second domain is located between said first and third domains.
- 15
13. A probeset for detecting Kappa immunoglobulin light chain mRNA or corresponding heteronuclear RNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 401 through 416, inclusive.
- 20

WO 02/22874

PCT/US01/28014

14. A probeset for detecting Lambda immunoglobulin light chain mRNA or corresponding heteronuclear RNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 501 through 509, 511-513, and 515.
- 5 15. A probeset for detecting cytomegalovirus (CMV) immediate early RNA and /or corresponding mRNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 221 through 241
- 10 16. A probeset for detecting Epstein Barr virus (EBV) early RNA, RNA 1 and RNA 2, (EBER) wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 51 through 54.
- 15 17. A probeset for detecting Human Alu repetitive satellite genomic DNA sequences wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 301 and 302.

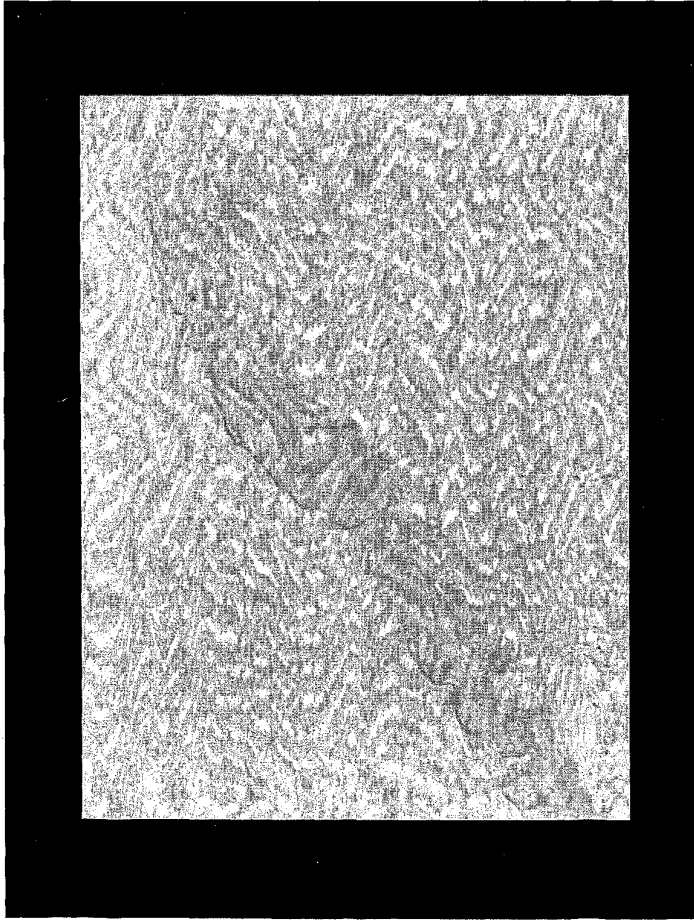
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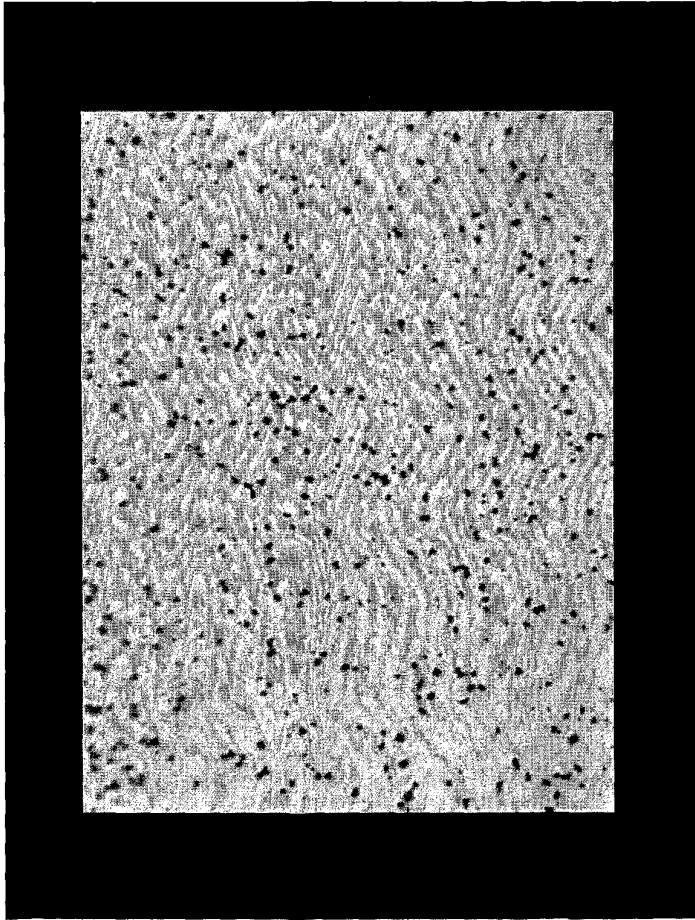
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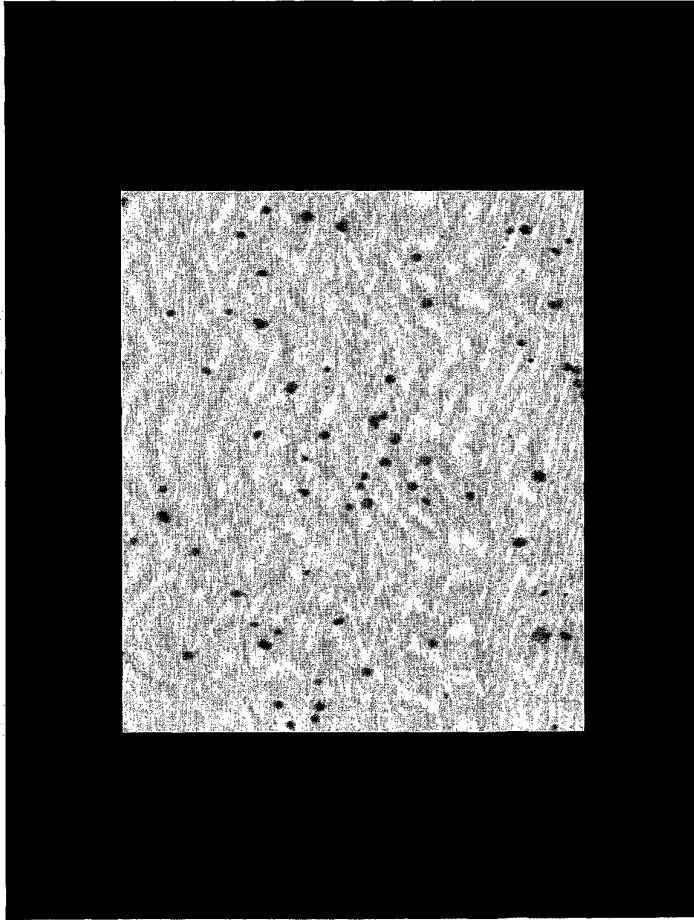
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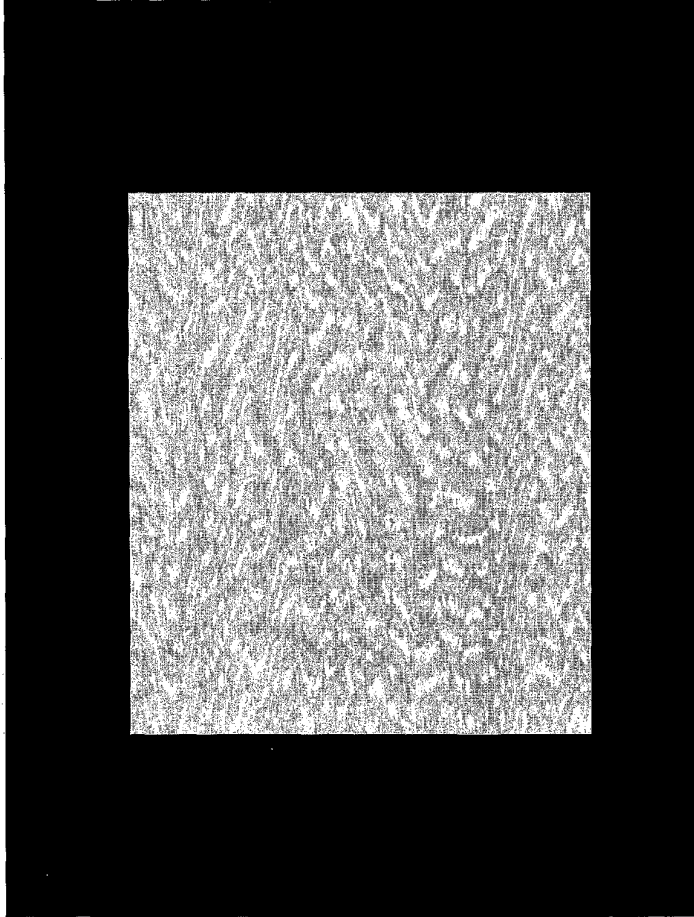
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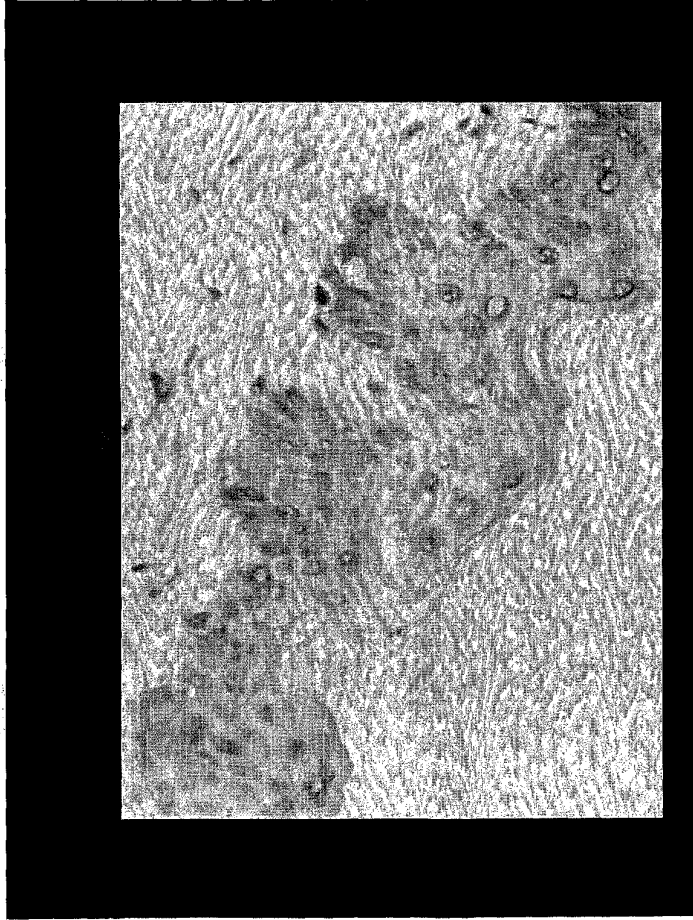
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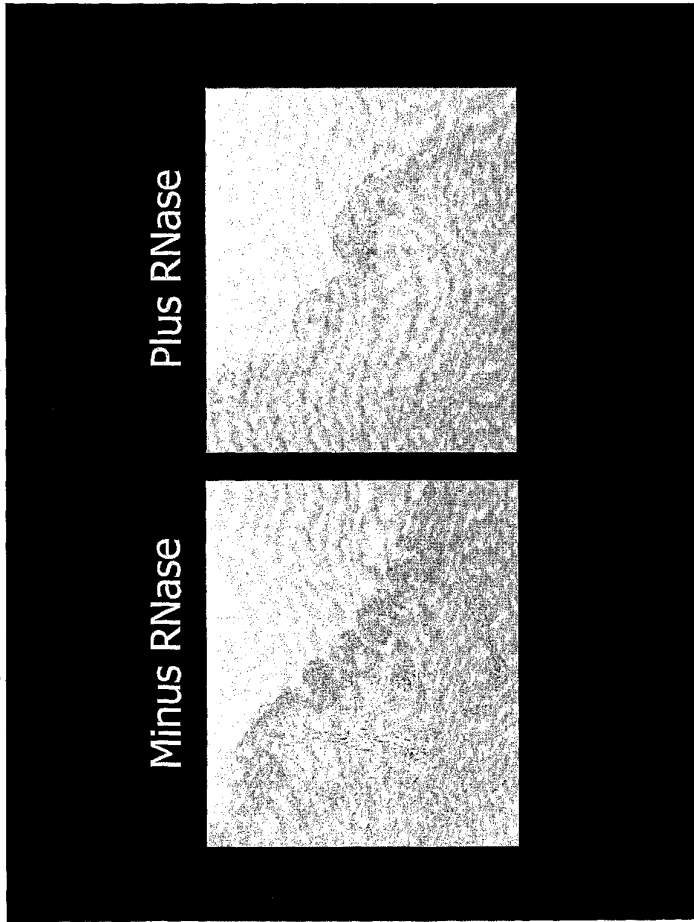


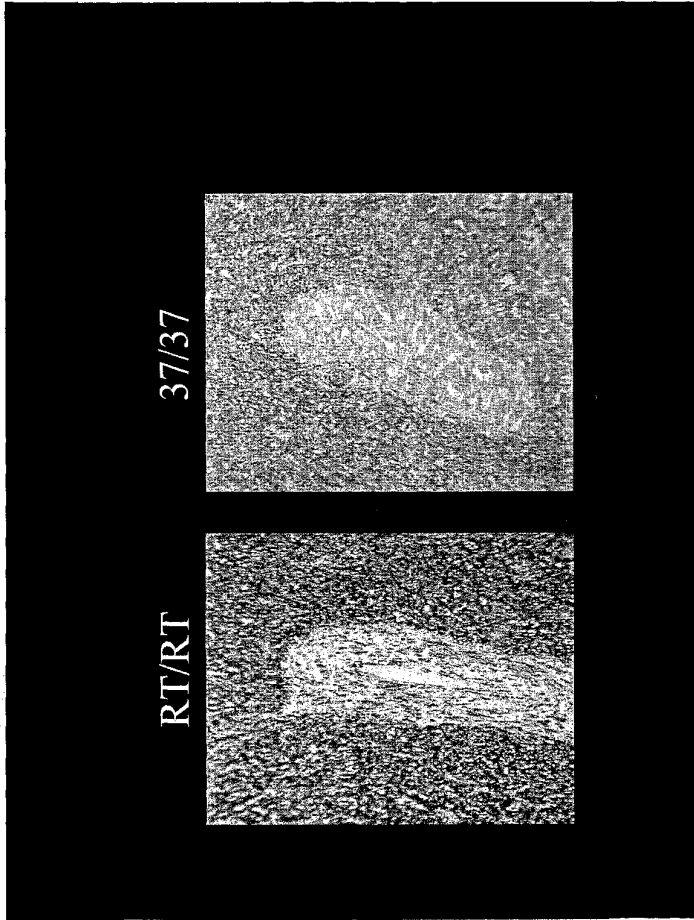


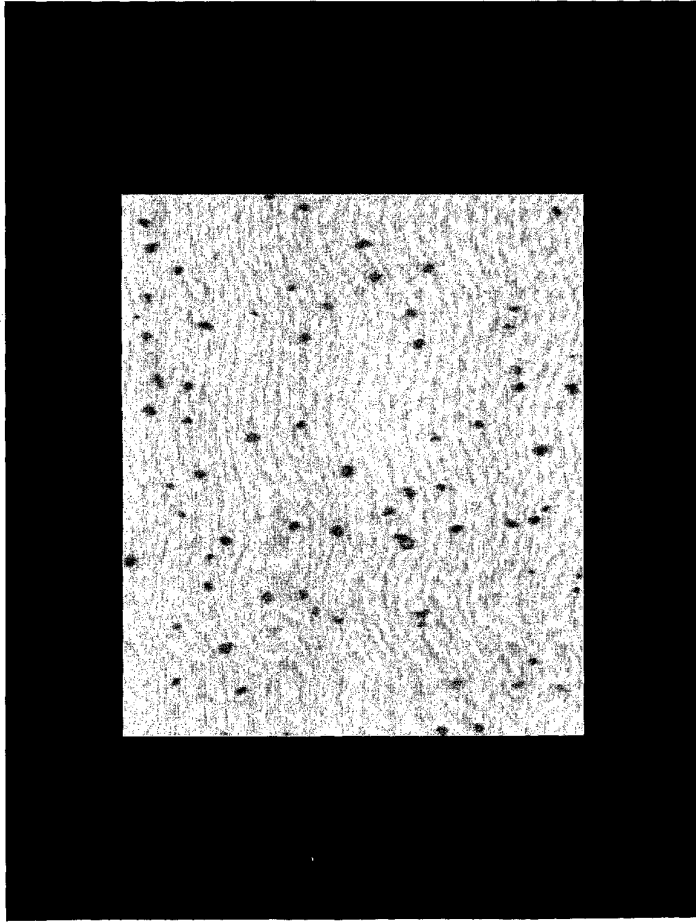


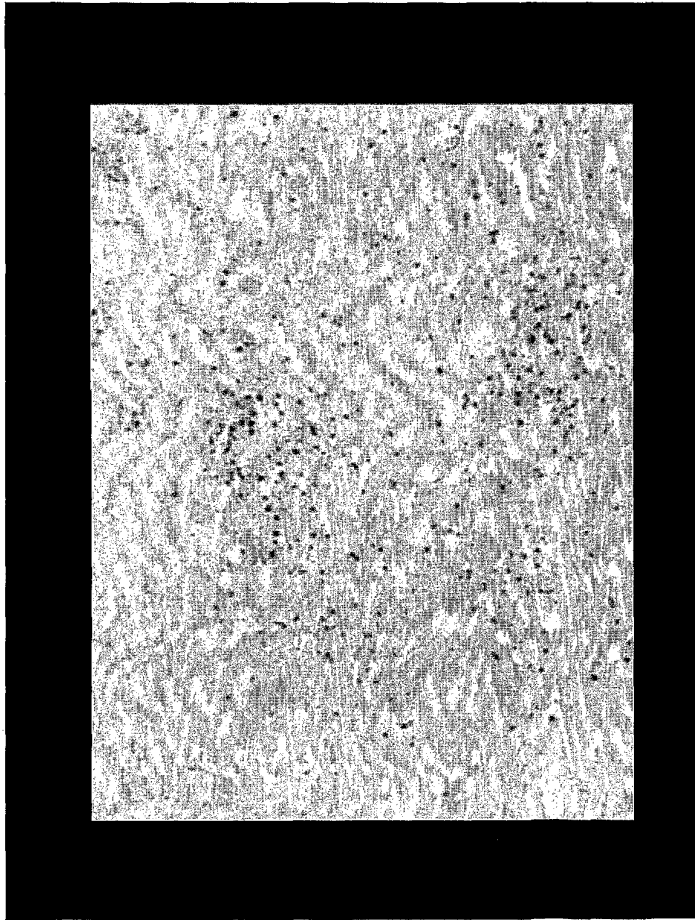


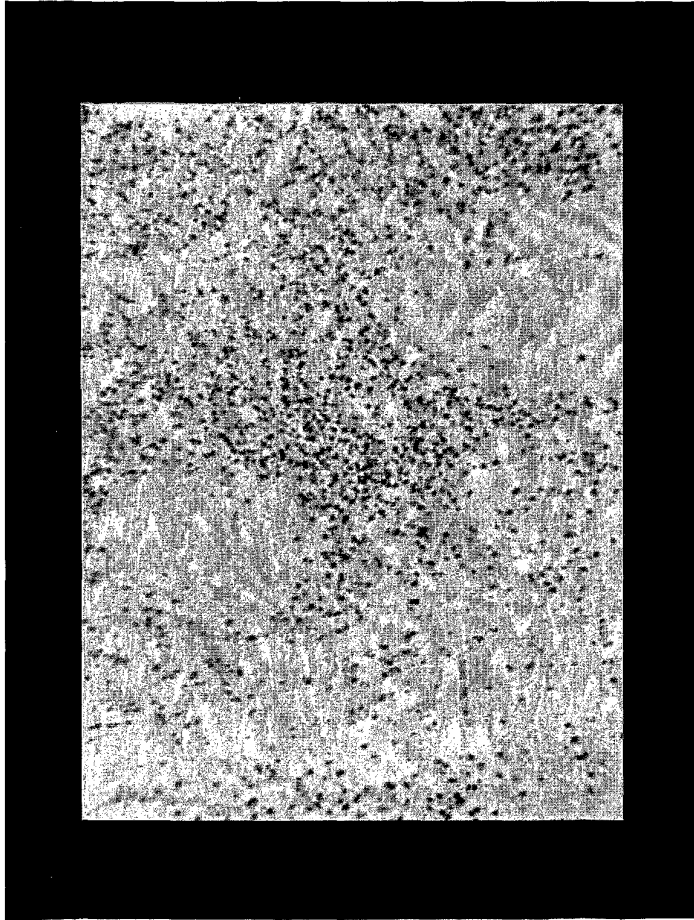


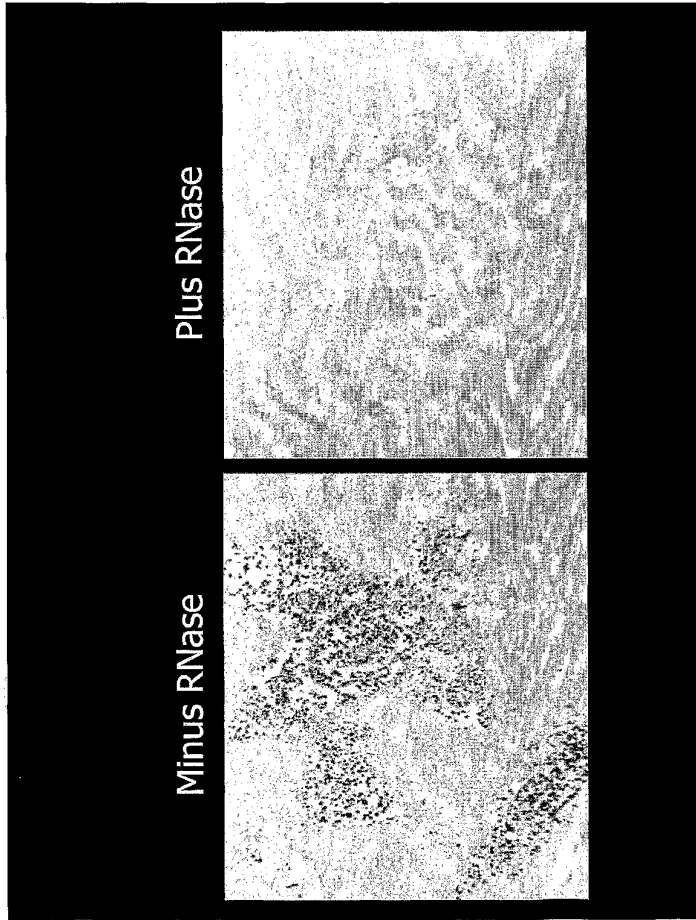


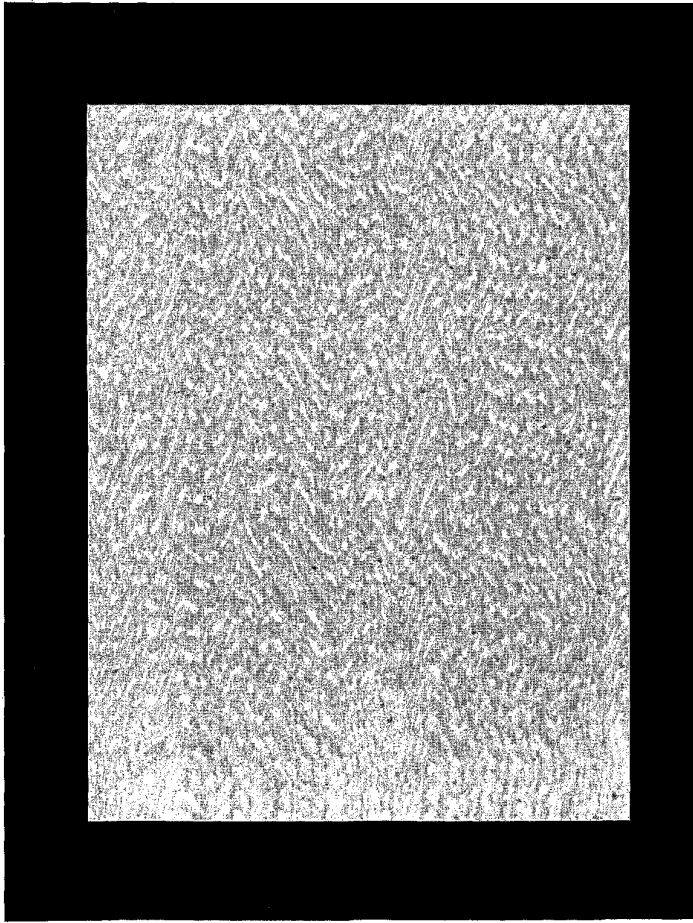


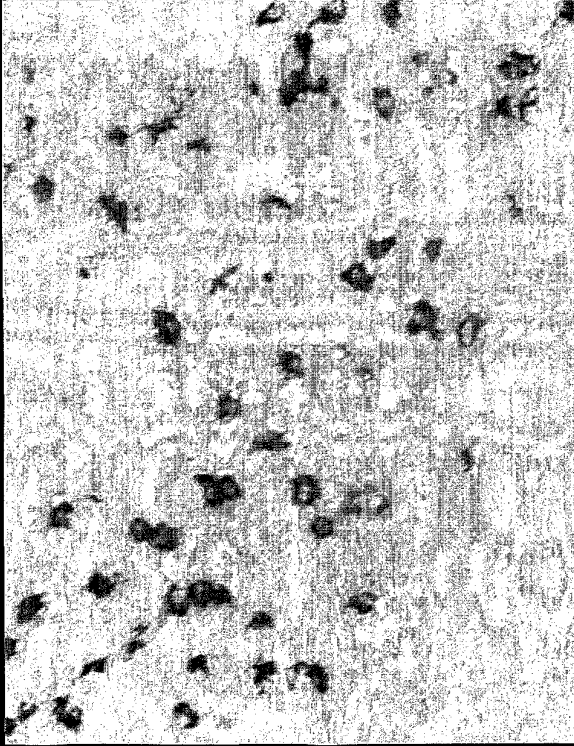


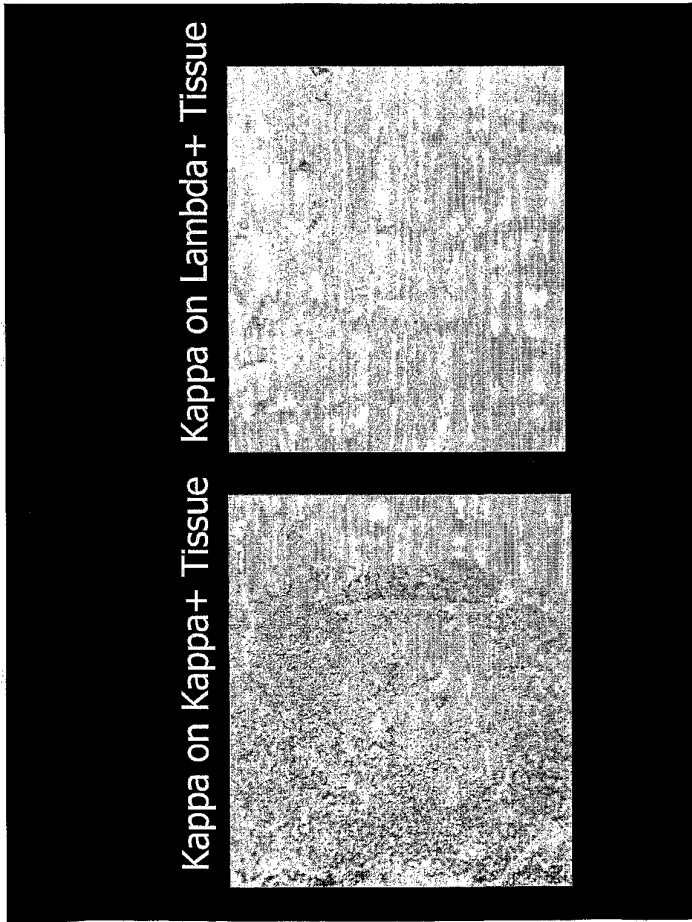


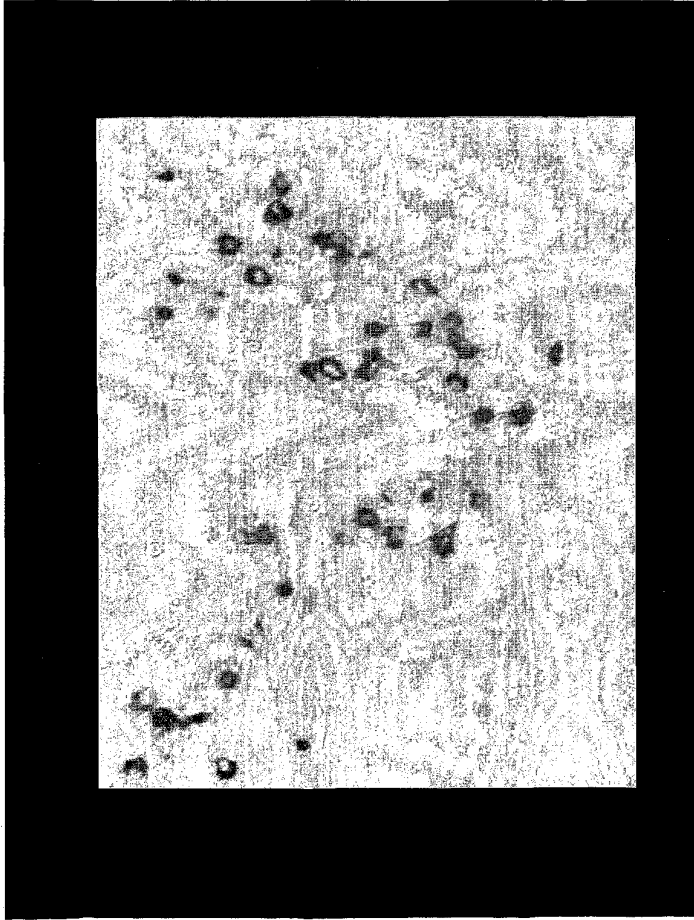


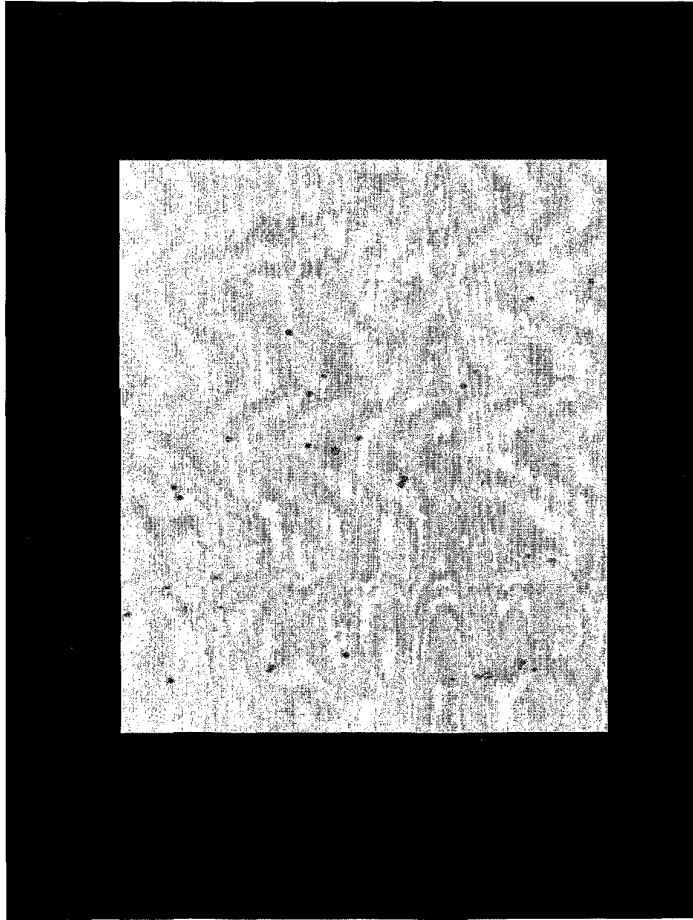


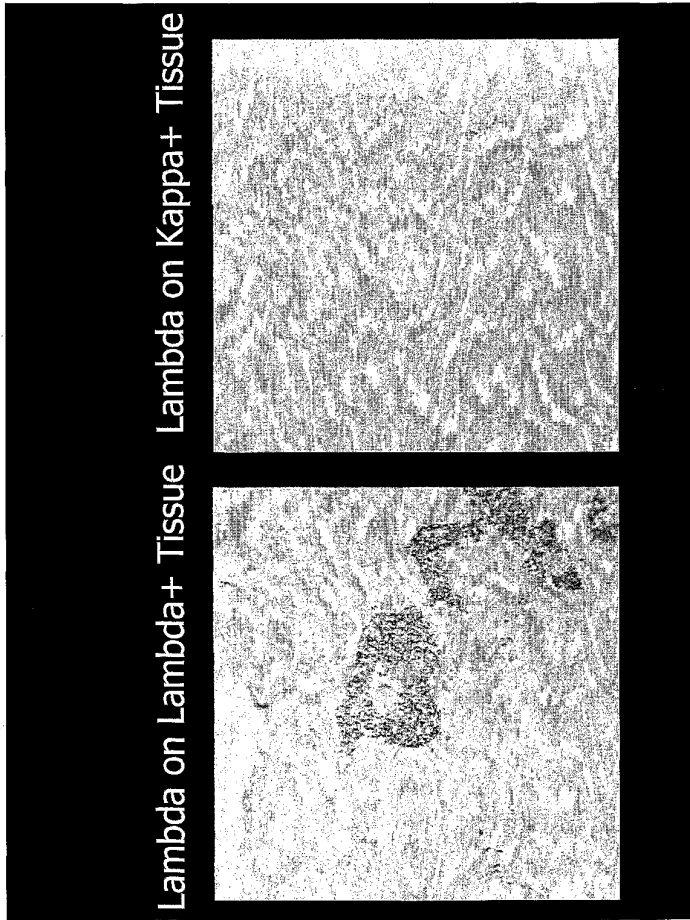


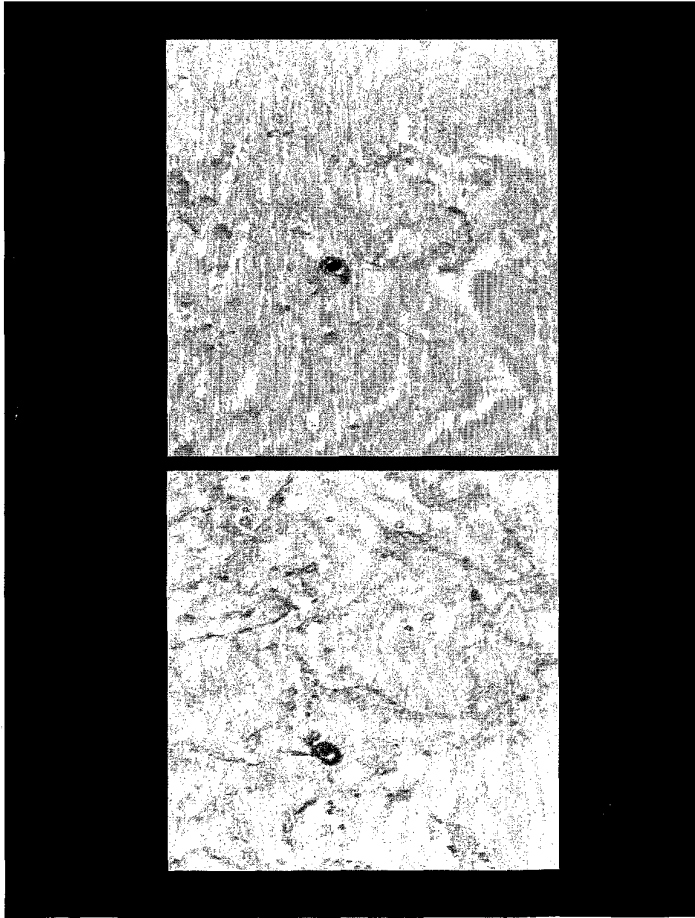


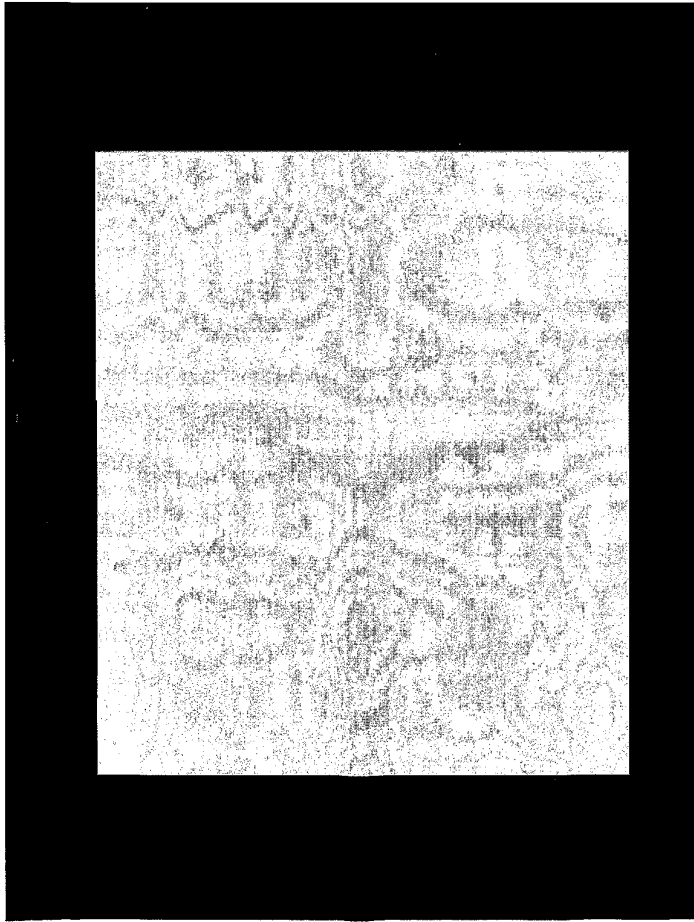


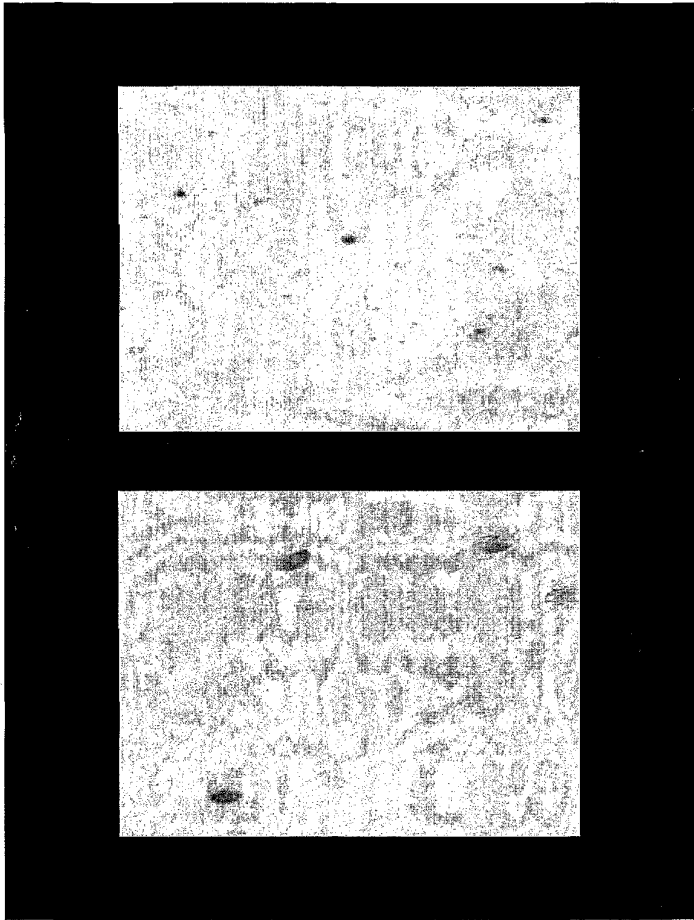












WO 02/22874

PCT/US01/28014

## SEQUENCE LISTING

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WO 02/22874

PCT/US01/28014

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WO 02/22874

PCT/US01/28014

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WO 02/22874

PCT/US01/28014

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<210> 15  
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WO 02/22874

PCT/US01/28014

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<210> 23

WO 02/22874

PCT/US01/28014

<211> 60  
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WO 02/22874

PCT/US01/28014

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<210> 30  
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WO 02/22874

PCT/US01/28014

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WO 02/22874

PCT/US01/28014

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<210> 39  
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WO 02/22874

PCT/US01/28014

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Oligonucleotide probe

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Oligonucleotide probe

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WO 02/22874

PCT/US01/28014

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WO 02/22874

PCT/US01/28014

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<210> 53  
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<210> 54  
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WO 02/22874

PCT/US01/28014

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<210> 58
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<210> 59
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<210> 60
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<210> 61

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WO 02/22874

PCT/US01/28014

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WO 02/22874

PCT/US01/28014

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<210> 66  
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<210> 68  
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Oligonucleotide probe

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WO 02/22874

PCT/US01/28014

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Oligonucleotide probe

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<210> 72  
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<220>  
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Oligonucleotide probe

<400> 72  
gaaaaagggt cagaggcca aggatgggag 30

<210> 73  
<211> 30  
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<220>  
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Oligonucleotide probe

<400> 73  
agatgagctg gaggaccgca atagggtag 30

<210> 74  
<211> 30  
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<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 74  
gcataattaa agcoaaggag gaggagggg 30

<210> 75  
<211> 30  
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<213> Artificial Sequence

WO 02/22874

PCT/US01/28014

<220>  
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Oligonucleotide probe

<400> 75  
cctgsgtgag gagggtgagg agcagcagag 30

<210> 76  
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<212> DNA  
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<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 76  
agaccagac acggaggcag gctgagtcag 30

<210> 77  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 77  
tgttggttcc agtcaggag atggtgatcg 30

<210> 78  
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Oligonucleotide probe

<400> 78  
taaatcatga tttgggggc tttgctggg 30

<210> 79  
<211> 30  
<212> DNA  
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Oligonucleotide probe

<400> 79  
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<210> 80

WO 02/22874

PCT/US01/28014

<211> 30  
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Oligonucleotide probe

<400> 80  
aataatcagc ctgctcctca gcctggagcc 30

<210> 81  
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Oligonucleotide probe

<400> 81  
ggtcctccg ccgaaaacca cagtgtaact 30

<210> 82  
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<400> 82  
ttatgagaca caccagtgtg gcctgttgg 30

<210> 83  
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<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 83  
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<210> 84  
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<220>  
<223> Description of Artificial Sequence:  
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<400> 84

WO 02/22874

PCT/US01/28014

atgcgtsacc tggcagctgt agcttctgtg 30

<210> 85  
<211> 30  
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Oligonucleotide probe

<400> 85  
attctgtagg ggcactgtc ttctccagg 30

<210> 86  
<211> 30  
<212> DNA  
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<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 86  
octcccctgg gatcctgcag ctctagtctc 30

<210> 87  
<211> 30  
<212> DNA  
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Oligonucleotide probe

<400> 87  
tgagggttta ttgagtgag ggagaagggc 30

<210> 88  
<211> 25  
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Oligonucleotide probe

<400> 88  
ggaggtcaaa acagcgtgga tggcg 25

<210> 89  
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WO 02/22874

PCT/US01/28014

Oligonucleotide probe

<400> 89  
gaggctggat cggccccgt gtctt 25

<210> 90  
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Oligonucleotide probe

<400> 90  
aatccgcggt ccaatgcacc gttcc 25

<210> 91  
<211> 25  
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<400> 91  
taaaaactgc gggcactggg gacgg 25

<210> 92  
<211> 25  
<212> DNA  
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Oligonucleotide probe

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accgagatt cgcgtggaga tocca 25

<210> 93  
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<400> 93  
gagcaaggag ctgccgagcg accat 25

<210> 94  
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WO 02/22874

PCT/US01/28014

<220>  
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<400> 94  
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<210> 95  
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<400> 95  
ttccaaatgc gtcagcggtg caagc 25

<210> 96  
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<400> 96  
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<210> 97  
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Oligonucleotide probe

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ccctccaccg ttaacagcac cgcaa 25

<210> 98  
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Oligonucleotide probe

<400> 98  
ttggtcacgg gtgtctcggg cctaa 25

<210> 99

WO 02/22874

PCT/US01/28014

<211> 25  
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Oligonucleotide probe

<400> 99  
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<210> 100  
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<400> 100  
tcggggttct cgttgcaatc ctgg 25

<210> 101  
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<400> 101  
atctcgatgc cccgctcaca tgcaa 25

<210> 102  
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<400> 102  
tgccgcacca tgcctactcg aaact 25

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<400> 103

WO 02/22874

PCT/US01/28014

gttagcggcg cccttgctca catca 25  
<210> 104  
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tgcagatctc ctcaatggcg cgctt 25  
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cctcatctga ctctcggcg atggc 25  
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WO 02/22874

PCT/US01/28014

Oligonucleotide probe

<400> 108  
 ggytgggtgc tcttgctcc agagg 25

<210> 109  
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<400> 109  
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<210> 110  
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<400> 110  
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<210> 111  
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<400> 111  
 ccacagacac cgtcctcacc acccg 25

<210> 112  
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 Oligonucleotide probe

<400> 112  
 ggctacagcc acacacgtct cctccc 26

<210> 113  
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WO 02/22874

PCT/US01/28014

<220>  
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<400> 113  
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<210> 114  
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<400> 114  
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<210> 115  
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<400> 115  
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<210> 116  
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<400> 116  
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<210> 117  
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<400> 117  
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<210> 118

WO 02/22874

PCT/US01/28014

<211>  
<212> DNA  
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<223> Description of Artificial Sequence:  
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<400> 118  
CTATTTTATACCTTTATATTCATATTTTATCT CGGGAGGCGGAGGTTGCAGTGAGCC 57

<210> 119  
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<400> 119  
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<210> 120  
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<212> DNA  
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<400> 120  
CTATTTTATACCTTTATATTTCT GACCTCGGTCGGTAGCACCCGCAC TACTATTTTATACCT 62

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<400> 121  
CTATTTTCTTT CGAGGCGGCGGATCACCTGAGGTC TTCCTTTTATCTT 49

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Oligonucleotide probe

WO 02/22874

PCT/US01/28014

<400> 122  
CTATTTTATACTTTATATTCTT CGAGGCGGGCGGATCACCTGAGGTC ACTATTTTATACTT  
61

<210> 123  
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<400> 123  
CTATTTTCTATTTTCTTTT  
21

<210> 124  
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Oligonucleotide probe

<400> 124  
CTATTTTATACTTTTATATTTCATATTTTATCT  
32

<210> 125  
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Oligonucleotide probe

<400> 125  
CTATTTTATACTTTATATTCTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNACTATTTTATACTT  
61

<210> 126  
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<212> DNA  
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<220>  
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Oligonucleotide probe

<400> 126  
CTATTTTCTTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTTCTTTTATCTT  
49

## 【国際公開パンフレット(コレクトバージョン)】

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
21 March 2002 (21.03.2002)

PCT

(10) International Publication Number  
WO 02/022874 A2

- (51) International Patent Classification: C12Q 1/68 (74) Agent: JONES, Huw, R.; Veniana Medical Systems, Inc., 1910 Innovation Park Drive, Tucson, AZ 85737 (US).
- (21) International Application Number: PCT/US01/28014
- (22) International Filing Date: 6 September 2001 (06.09.2001) (81) Designated States (national): AU, CA, JP, MX, US.
- (25) Filing Language: English (84) Designated States (regional): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).
- (26) Publication Language: English Published: — without international search report and to be republished upon receipt of that report
- (30) Priority Data: 60/233,177 15 September 2000 (15.09.2000) US (48) Date of publication of this corrected version: 3 April 2003
- (71) Applicant (for all designated States except US): VENTANA MEDICAL SYSTEMS, INC. [US/US]; 3865 N. Business Center Drive, Tucson, AZ 85705 (US).
- (72) Inventors; and (75) Inventors/Applicants (for US only): UTERMÖHLEN, Joseph, G. [US/US]; 5357 East 19th Street, Tucson, AZ 85711 (US). CONNAUGHTON, John [US/US]; 9117 Huntmaster Road, Laytonsville, MD 20882 (US).
- (15) Information about Correction: see PCT Gazette No. 14/2003 of 3 April 2003, Section II
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: OLIGONUCLEOTIDE SEQUENCE FORMULA FOR LABELING OLIGONUCLEOTIDE PROBE AND PROTEINS FOR IN-SITU ANALYSIS

## Oligonucleotide Probe Design

Common Signal Detection Region Unique Target Detection Region

5' - [ ] - 3'

Alu301 5'-CTATTTTCIATTTCTATTTCTATTTCT-CGAGGCGGGCGGATCACCTGAGGTC-3'

Alu302 5'-CTATTTTCIATTTCTATTTCTATTTCT-CGGGAGGCGGAGGTTGCAGTGAGCC-3'

(57) Abstract: The present invention provides oligonucleotide probes and oligonucleotide probe collections and protein labeling for detecting or localizing a plurality nucleic acid target genes or antigens within a cell or tissue sample. Specifically, the provides collections of oligonucleotide probe for use in *in situ* hybridization analyses in which each probe has a label-domain with the sequence formulas of (CTATTTT)<sub>n</sub>, (AAAATAG)<sub>n</sub>, or (TTTTATC)<sub>n</sub>, or (GATAAAA)<sub>n</sub>, in which all cases "n" would equal 1 or greater. The present invention provides collections or "cocktails" of oligonucleotide probes for detecting or localizing specific nucleic acid target genes within a cell or tissue sample. The cocktails are useful for detecting the following: the Kappa gene (SEQ ID NOS: 1-16 inclusive); the Lambda gene (SEQ ID NOS: 501-509, 511-513, and 515); the CMV (cytomegalovirus) gene (SEQ ID NOS: 221-241 inclusive); EBV1R (Epstein-Barr RNA) gene (SEQ IN NOS: 51-54 inclusive); Alu (SEQ IN NOS: 55-56); PolyA (SEQ ID NO: 57); and the detection tail (SEQ ID NO: 330).

WO 02/022874 A2

WO 02/022874

PCT/US01/28014

OLIGONUCLEOTIDE SEQUENCE FORMULA FOR LABELING  
OLIGONUCLEOTIDE PROBES AND PROTEINS FOR *IN SITU* ANALYSIS**BACKGROUND OF THE INVENTION****1. Field of the Invention**

This invention relates to oligonucleotide probes and collections of oligonucleotide probes for detecting or localizing nucleic acid genes targets within a cell or tissue sample. In particular, the invention relates to collections of oligoprobes.

**2. Background of the Invention**

*In situ* analysis includes *in situ* hybridization and immunohistochemistry. *In situ* hybridization (ISH) employs labeled DNA or RNA probe molecules that are anti-sense to a target gene sequence or transcript to detect or localize targeted nucleic acid target genes within a cell or tissue sample. ISH has proven to be a useful tool in a number of biomedical fields, including developmental biology, cell biology, and molecular biology. ISH has been used, for example, to diagnose genetic disorders, map genes, study gene expression, and localize sites of target gene expression.

Typically, ISH is performed by exposing a cell or tissue sample immobilized on a glass slide to a labeled nucleic acid probe which is capable of specifically hybridizing to a given target gene in the cell or tissue sample (*In Situ Hybridization: Medical Applications* (G. R. Coulton and J. de Bellerche, eds., Kluwer Academic Publishers, 1992); *In Situ Hybridization: In Neurobiology; Advances in Methodology* (J. H. Eberwine, K. L. Valentino, and J. D. Barchas, eds., Oxford University Press, 1994); *In Situ Hybridization: A Practical Approach* (D. G. Wilkinson, ed., Oxford University Press, 1992)). The hybridization of labeled probe molecules to nucleic acids in the cell or tissue sample can then be detected using, for example, radioactive-based direct detection methods, fluorescence-based direct detection methods, or indirect detection methods based on the binding of a fluorescence-labeled protein binding to a hapten such as BrdU, digoxigenin-labeled or biotin-labeled nucleotides incorporated into probes. Hapten-based methods have been further extended to include those molecules to be bonded by binding protein-enzyme conjugates such as antibody-enzyme-conjugates and colorimetric based detection chemistry. In addition, several target genes can be simultaneously analyzed by exposing a cell or tissue sample to a plurality of nucleic acid probes that have been

- 1 -

SUBSTITUTE SHEET (RULE 26)

WO 02/022874

PCT/US01/28014

labeled with a plurality of different nucleic acid tags. For example, a plurality of nucleic acid probes can be labeled with a plurality of fluorescent compounds having different emission wavelengths, thereby permitting simultaneous multicolored analysis to be performed in a single step on a single target cell or tissue sample.

A significant problem associated with incorporation of labeled nucleotides into oligonucleotide probes is that the conjugation moieties that are attached to the nucleotide usually interfere with the formation of Watson-Crick base pairing, thus negatively affecting the hybridization of the probe to its target. This has been seen with use of label attached via N4-substituted cytosine nucleotides, because of steric hinderance and the expected shift to the less reactive state of a secondary amine (as seen with N4 labeled cytosine), as compared to the natural G-C bond formed with an unsubstituted cytosine (a primary amine). Any small change or interference with G-C bonding in a small oligonucleotide (25 to 50 bases) can reduce the ability of these oligos to hybridize with the intended targeted sequence.

There remains a need in the art to develop suitable probes designs for incorporating labeled nucleotides in oligonucleotide probes. We demonstrate that a few artificial sequences are viable alternatives for probe labeling and also work both singly and in complex oligonucleotide probe mixtures for detecting or localizing nucleic acid target genes within a cell or tissue sample. The development of such generic sequences and labeling strategy for probe collections has wide application in the medical, genetic, and molecular biological arts.

This interference due to labeling chemistry and hybridization stringency and kinetics is solved herein by designing the oligo to have at least two distinct functional domains, one domain or sequence to be gene specific and involved in the base pair formation, and the second domain to be an artificial, non-specific sequence (in reference to the sample's genome) comprised of spacing nucleotides and the labeled nucleotide. These elements are positioned so that these label-nucleotides are more accessible as haptens for binding proteins (immunoglobulin or avidin(s)) and thus do not interfere with Watson-Crick base pairing in the gene-specific domain.

WO 02/022874

PCT/US01/28014

**SUMMARY OF THE INVENTION**

The present invention provides a novel strategy to incorporate label into oligonucleotide probes and labeled oligonucleotide probe collections for detecting or localizing nucleic acid target genes within a cell or tissue sample. In particular, the invention relates to non-gene-specific sequences using sequence formulas for making repetitive polymers of such sequences which can be incorporated into collections of oligonucleotide probes for use in *in situ* hybridization analyses. In addition, using labeled synthetic oligonucleotide polymers, based on sequence formulas, when conjugated to binding proteins, i.e. immunoglobulins, is a very effective and controlled process for labeling such proteins used in immunohistochemical analysis. The present invention provides collections or "cocktails" of oligonucleotide probes for detecting or localizing specific nucleic acid target genes within a cell or tissue sample. The cocktails are useful for detecting the following: the Kappa gene (SEQ ID NOS: 1-16 inclusive); the Lambda gene (SEQ ID NOS: 501-509, 511-513, and 515); the CMV (cytomegalovirus) gene (SEQ ID NOS: 221-241 inclusive); EBER (Epstein-Barr early RNA) gene (SEQ ID NOS: 51-54 inclusive); A<sub>u</sub> (SEQ ID NOS: 55-56); PolyA (SEQ ID NO: 57); and the detection tail (SEQ ID NO:330).

The invention is directed to an oligonucleotide label-domain comprising the sequence (CTATTT)<sub>n</sub> and its complement (AAAATAG)<sub>n</sub> wherein "n" is at least 1.

The invention is also directed to an oligonucleotide probe having at least two distinct functional domains, a first domain comprising the label-domain of claim 2, and a second domain comprising a gene-specific target sequence.

The invention is also directed to a probeset for detecting Kappa immunoglobulin light chain mRNA or corresponding heteronuclear RNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 401 through 416, inclusive.

The invention is also directed to a probeset for detecting Lambda immunoglobulin light chain mRNA or corresponding heteronuclear RNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 501 through 509, 511-513, and 515.

The invention is also directed to a probeset for detecting cytomegalovirus (CMV) immediate early RNA and/or corresponding mRNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 221 through 241.

- 3 -

**SUBSTITUTE SHEET (RULE 26)**

WO 02/022874

PCT/US01/28014

The invention is also directed to a probeset for detecting Epstein Barr virus (EBV) early RNA, RNA 1 and RNA 2, (EBER) wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 51 through 54.

The invention is also directed to a probeset for detecting Human Alu repetitive satellite genomic DNA sequences wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 301 and 302.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a generic probe structure of the two-domain probe design. This is the oligonucleotide design used for the probes in the gene specific cocktails described in the following examples. Each probe is composed of two domains: a 5' labeling domain and a 3' target gene target gene-specific domain. The labeling domain consists of this specific sequence (CTATTTT)<sub>n</sub>, wherein each cytosine may be labeled with a fluorophore or a cytosine-hapten conjugate, the hapten being fluorescein in this embodiment. This illustration specifically shows nucleic acid sequences for the 301 (SEQ ID NO: 55) and 302 (SEQ ID NO: 56) probes, each of which possesses target gene gene-specific domains corresponding to human repetitive Alu sequences and labeling domains having a fluorescein hapten.

Figure 2 illustrates the results obtained for *in situ* hybridization (ISH) analysis of human skin tissue using a probe comprising the labeling domain (330 probe; SEQ ID NO: 58). The absence of a detectable signal indicates that the sequence formula, (CTATTTT)<sub>n</sub>, of the labeling domain common to the oligonucleotides used in these ISH examples is non-specific, and non-reactive in its ability to form Watson-Crick base pairing with human nucleic acid sequences because it does not hybridize.

Figure 3 illustrates the results obtained for ISH analysis of human skin tissue using a probe comprising the labeling domain and a poly d(T) target gene-specific domain (320 probe; SEQ ID NO: 57). The presence of a detectable signal localized to the cytoplasm indicates that this probe is capable of specifically hybridizing to polyadenylated region of messenger RNA.

Figures 4A-4B illustrate the results obtained for ISH analysis of human skin tissue using the 320 probe, wherein the tissue sample was not treated with ribonuclease A prior

WO 02/022874

PCT/US01/28014

to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The decrease in detectable signal in (B) indicates that this probe specifically hybridizes to polyadenylated region common to messenger RNA. .

Figures 5A-5B illustrate the results obtained for ISH analysis of human spleen tissue using the 320 probe, wherein the hybridization and stringency wash were performed at room temperature (A), or at 37°C (B). This result illustrates that the intensity of color is related to the stringency of hybridization conditions, with the more intense color indicating less stringent conditions.

Figure 6 illustrates the results obtained for ISH analysis of the human Raji cell line using the 320 probe. This shows that this probe design also is functional with embedded cell lines as well as embedded tissue.

Figure 7 illustrates the results obtained for ISH analysis of the human Raji cell line using a probe collection consisting of the 301 and 302 probes.

Figure 8 illustrates the results obtained for ISH analysis of the human HT cell line using a probe collection consisting of the 301 and 302 probes.

Figure 9 illustrates the results obtained for ISH analysis of a rat cell line using a probe collection consisting of the 301 and 302 probes. The absence of a detectable signal indicates that this probe collection is specific for human nucleic acid sequences.

Figure 10 illustrates the results obtained for ISH analysis of an Epstein-Barr virus (EBV)-negative human HT cell line using a probe possessing a target gene-specific domain corresponding to EBV EBER nuclear RNA [SEQ ID NO: 51 through SEQ ID NO: 54].

Figure 11 illustrates the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54].

Figure 12 illustrates the results obtained for ISH analysis of human tonsil tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54].

Figures 13A-13B illustrate the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through

WO 02/022874

PCT/US01/28014

SEQ ID NO:54, wherein the tissue sample was not treated with ribonuclease A prior to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The decrease in detectable signal in (B) indicates that this probe specifically hybridizes to human EBER 1 and EBER 2 nuclear RNA.

Figure 14 illustrates the results obtained for ISH analysis of kappa light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NO:15].

Figure 15 illustrates the results obtained for ISH analysis of lymphoma tissues using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin kappa light chain mRNA [SEQ ID NOS: 2 - 4, SEQ ID NOS:7-12, SEQ ID NOS: 14, 15]. The lymphoma tissue in (A) over expresses the kappa light chain and the tissue in (B) over expresses the lambda light chain. The absence of a detectable signal in (B) indicates that the kappa light chain probe collection is specific to kappa light chain mRNA.

Figure 16 illustrates the results obtained for ISH analysis of lambda light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain variable region mRNA [SEQ ID NOS:19 through 29].

Figure 17 illustrates the results obtained for ISH analysis of a lambda light chain-positive human RPMI 8226 cell line using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NOS:19 through 29].

Figures 18A-18B illustrate the results obtained for ISH analysis of human lymphoma tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NOS:19 through 29]. The tissue in (A) over expresses the lambda light chain and the tissue in (B) over expresses the kappa light chain. The absence of a detectable signal in (B) indicates that the lambda light chain probe collection is specific to human lambda light chain mRNA.

Figure 19 illustrates the results obtained for ISH analysis of cytomegalovirus (CMV)-positive human lung tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA [

WO 02/022874

PCT/US01/28014

SEQ ID NOS:30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50] [ CMV infected cell ]].

Figure 20 illustrates the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has not been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early mRNA [ SEQ ID NOS:30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50].

Figures 21A-21B illustrate the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA [ SEQ ID NOS:30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50]. The tissue in (A) is shown at a magnification of 40X and the tissue in (B) is shown at a magnification of 20X.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides oligonucleotide probes and oligonucleotide probe collections for detecting or localizing nucleic acid target genes within a cell or tissue sample. In particular, the invention relates to collections of oligonucleotide probes for use in *in situ* hybridization analyses.

More specifically, this invention relates to the use of specific sequence formulas for nucleotide polymers or label-domains to attach a detectable moiety ( a label) to oligonucleotide probes or proteins. The specific utility of these sequences or derivatives thereof, is the inert or non-reactive characteristic that does not hybridize to human DNA or RNA at a detectable level under standard stringency of hybridization conditions. These label-domains or polymers were demonstrated to be useful generic sequences for incorporation into oligonucleotide probes for detecting gene-specific sequences within cells or tissue samples in *in situ* hybridization analyses. Additionally, this inert set of sequences are useful for attaching a label to immunoglobulins or other proteins for detecting haptens and antigens in immunohistochemical analyses.

As used herein, the terms "probe" or "oligonucleotide probe" refers to a nucleic acid molecule used to detect a complementary nucleic acid target gene.

As used herein, the term "hybridization" refers to the process whereby complementary nucleic acid sequences join to form a double-stranded nucleic acid

WO 02/022874

PCT/US01/28014

molecule. By labeling the target nucleic acid molecule with, for example, a radioactive or fluorescent tag, interactions between probe and target genes can be detected.

The oligonucleotide probes and oligonucleotide probes of the collections of the present invention are synthesized using conventional methods. See e.g., *Methods in Molecular Biology, Vol 20: Protocols for Oligonucleotides and Analogs* 165-89 (S. Agrawal, ed., 1993); *Oligonucleotides and Analogues: A Practical Approach* 87-108 (F. Eckstein, ed., 1991).

In a preferred embodiment of the present invention, oligonucleotide probes possess two distinct domains: a 5' (or labeling) domain and a 3' (or gene-specific target) domain (See Figure 1A). In more preferred embodiments of the present invention, the oligonucleotide probe possesses a labeling domain which consists of the sequence (CTATTTT)<sub>n</sub>. Other embodiments are also demonstrated herein, including a triple-domain embodiment having two terminal labeling domains, and a central gene-specific target domain. Specifically, SEQ ID NOS: 125-126 depict this labeling scheme. Yet a further preferred embodiment of a labeling domain is TC(TTTTATC)<sub>n</sub> or its complement. This sequence is predicted to be as unique as the (CTATTTT)<sub>n</sub> label-domain. The oligonucleotide probes of the present invention are labeled so that hybridization between said probes and target nucleic acids in a particular cell or tissue can be detected. Labels that are acceptable for use in *in situ* hybridization (ISH) analysis are known to those with skill in the art. Such labels permit interactions between probe and target genes to be detected using, for example, radioactive-based direct detection methods, fluorescence-based direct detection methods, digoxigenin-labeled or biotin-labeled probes coupled with fluorescence-based detection methods, or digoxigenin-labeled or biotin-labeled probes coupled with antibody-enzyme-based detection methods. In preferred embodiments of the present invention, oligonucleotide probes are labeled with fluorescein. In more preferred embodiments of the present invention, the oligonucleotide probe possesses a labeling domain which consists of the sequence (CTATTTT)<sub>n</sub>, wherein the cytosine nucleotides may be labeled with a fluorophore for direct detection, or a hapten for indirect detection. In either, the fluorescein-cytosine nucleotide conjugate and the fluorescein molecule is linked at the N4 position of cytosine through an OBEA linkage (See Mishra *et al.*, U.S. Patent No. 5,684,142, which is incorporated herein by reference). In a preferred embodiment, the density of fluorophore attached to the label-domain is at least 7 mole percent, preferably at least 10 mole

- 8 -

SUBSTITUTE SHEET (RULE 26)

WO 02/022874

PCT/US01/28014

percent, and most preferably at least 16 mole percent, when measured against the label-domain solely. For example, if probe 401 is considered (a 2-domain probe) it comprises a label-domain of 30 bases including a 3' terminal CT wherein the C is also labeled, the mole percent is  $5/30 = 16.7$  mole percent label. In the overall probe, the mole percent is 8.3.

In some embodiments of the present invention, several target genes are simultaneously analyzed by exposing a cell or tissue sample to a plurality of nucleic acid probes that have been labeled with a plurality of different nucleic acid tags. For example, a plurality of nucleic acid probes can be labeled with a plurality of fluorescent compounds having different emission wavelengths, thereby permitting simultaneous multicolored analysis to be performed in a single step on a single target cell or tissue sample.

The oligonucleotide probes and oligonucleotide probe collections of the present invention may be used in ISH analysis to detect or localize nucleic acid target genes within a cell or tissue sample. ISH may be performed as described, for example, in *In Situ Hybridization: Medical Applications* (G. R. Coulton and J. de Bellerche, eds., Kluwer Academic Publishers, 1992); *In Situ Hybridization: In Neurobiology; Advances in Methodology* (J. H. Eberwine, K. L. Valentino, and J. D. Barchas, eds., Oxford University Press, 1994); or *In Situ Hybridization: A Practical Approach* (D. G. Wilkinson, ed., Oxford University Press, 1992).

The preferred embodiment of the probes and probe collections of the present invention are best understood by referring to Figures 1-21 and Examples 1-2. The Examples, which follow, are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

#### EXAMPLE 1

##### Probe Collection Preparation

Probe collections consisting of a plurality of oligonucleotide probes of 55 to 60 bases in length were designed as follows. In this Example, each oligonucleotide probe possessed two distinct domains: a 5' (or labeling) domain and a 3' (or target gene-specific) domain (See Figure 1).

In this embodiment, the labeling domain consists of the sequence (CTATTT)<sub>n</sub>, wherein the cytosine nucleotide represents a fluorescein-cytosine nucleotide conjugate

WO 02/022874

PCT/US01/28014

and the fluorescein molecule is linked at the N4 position of cytosine through an OBEEA linkage.

The target gene-specific domain consists of a 25-30 base sequence that is complementary to a specific nucleic acid target gene. Oligonucleotide probes were designed to possess target gene-specific domains corresponding to the human immunoglobulin kappa light chain variable region (See Table 1; oligonucleotide probes 401-416), the human immunoglobulin lambda light chain variable region (oligonucleotide probes 501-515), human cytomegalovirus (CMV) sequences (oligonucleotide probes 221-241), human Epstein-Barr virus (EBV) EBER (Epstein-Barr early RNA) sequences (oligonucleotide probes 100A2, 100C2, 100A1, and 100B1), human repetitive Alu sequences (oligonucleotide probes 301 and 302), and poly d(T) (oligonucleotide probe 320).

#### EXAMPLE 2

##### Label-domain design: Alu repetitive sequence probe

Four probes all against the Alu human repetitive sequence were used to evaluate label-domain design. The probes numbered 301 (SEQ ID NO: 55), 301A (SEQ ID NO:116), 301A2/2 (SEQ ID NO: 121), and 301A3/2 (SEQ ID NO: 122) are shown in Table 1.

The four probes were evaluated at the concentrations of 100, 75, 50, and 25 ng/ml per mL of probe in the reaction, respectively. This hybridization analysis was done manually, using standard protocols. The target, paraffin-embedded cell line MBA MD 468 (Oncor INFORM™ Her-2/neu Control Slides, Cat. No. S8100, Level 1, available from Ventana Medical Systems, Inc., Tucson, AZ) was the target sample and was processed by removing paraffin by standard xylene methods. The tissue was subjected to Ventana's Protease 1 for 12 minutes at 50 degrees C as a 1:2 dilution with Ventana's APK buffer. The hybridization reaction was accomplished with the addition of probe diluent as 100 ul probe (25% formamide, 5% dextran sulfate, 2X SSC, 1% Triton) to a residual 100 ul volume of 2XSSC/Triton X-100. The slide was heated to 85 degrees C for 5 minutes and then incubated for 1 hr at 37 degrees C. Standard SSC washes followed for removing excess probe. The hybrids were detected with an antibody against FITC. The mouse antibody was detected colorimetrically using Ventana Enhanced Alkaline Phosphatase Blue Detection (cat# 760-061). Unless otherwise indicated, all reagents

WO 02/022874

PCT/US01/28014

were obtained from Ventana Medical Systems, Inc., Tucson, AZ. The results were observed by colorimetric detection using brightfield microscopy.

The results of these experiments were that signal intensity was a function of the total number of fluorescein haptens conjugated to the probe and signal was of the specific label-domain design. The greater the number of fluoresceins per probe molecule, the greater the signal observed. Comparison of design and placement of haptens on the probe showed that this was not a factor in signal intensity. The two probes that contained five fluoresceins, (301A3/2 (SEQ ID NO: 122) and 301 (SEQ ID NO:55) both yielded equivalent signal. These two probes yielded greater signal than seen for 301A2/2, a probe with a split label-domain design with four fluoresceins. The probe 301A2/2 yielded a signal greater than probe 301A a probe with a single label-domain design at the 5' end and with three fluoresceins.

### EXAMPLE 3

#### Label Domain Design: EBER probes

This experiment compared two label-domain designs and sequences to determine whether greater spacing between the fluorescein haptens improves the production of signal during probe detection steps during *in situ* hybridization analysis.

The tissue used was an EBV-infected human spleen tissue fixed in neutral buffered formalin paraffin embedded section of 4-micron thickness placed on silane plus glass microscope slides. The tissue sections were deparaffinized on a Ventana DISCOVERY™ machine, followed by a 6-min digestion with Ventana's Protease 1, at a temperature of 37 C. The probe was dissolved in hybridization buffer diluent at a concentration of 50 ng/mL as a 100 ul applied to an equal volume of 2 X SSC/ Triton X-100 residual volume left on the slide after prepared by the Ventana Medical Systems, Inc. automated ISH staining system, Discovery. The probe diluent-mixed with the residual volume on slide for 6 min at 37 C, then the solution was heated to 85C and held there for a total of 10 min. The slide was then taken to a 37C temperature and held at that temperature for 1 hour. All of these aqueous reactions on the slide were all done under a film of LIQUID COVERSLIP™, to prevent evaporative loss of water during processing. Each slide after hybridization was washed 3 times with 2X SSC/Triton solution, with a 6 min incubation between each wash, the slide volume being approximately 300 ul (+/- 10% vol). The hybrids were detected with an antibody against FITC. The mouse

- 11 -

SUBSTITUTE SHEET (RULE 26)

WO 02/022874

PCT/US01/28014

antibody was detected colorimetrically using Ventana Enhanced Alkaline Phosphatase Blue Detection (cat# 760-061).

The two oligonucleotide probes used for this study probe 100A1 (SEQ ID NO: 53) and 1002A32 (SEQ ID NO: 120). The two differences between these probes were the label-domain sequence and structure. The probe 100A1 label domain was 5' to gene target domain, contained 5 fluoresceins attached to cytosine residues via the OBEA linker, with the sequence formula of (CTATTTT)<sub>n</sub>CT (SEQ ID NO: 58). The label domain of the oligo probe 1002A32, was similar, (SEQ ID NO:125). Besides the different sequence the primary difference was that the fluoresceine labeled cytosines were spaced 10 bases apart compared to the oligo 100A1 the cytosine spacing was closer at 7 bases apart. The result of this comparison as deduced by H score analysis were that these oligonucleotide were equivalent as to the amount of signal generated on the slide. The data was that for 100A2, for the 368 cells analysed in a total of 3 fields the H score was 106, and for probe 1002A32 for the 345 cell analysed in three field the H score was 109. The H score is a spectrographic analysis done with microscope that factors into the score background to signal ratio on the tissue section to yield a relative comparison of total target specific signal on the slide. (See reference Giroud, F. Perrin C, and Simony Lafontaine, J.; Quantitative Immunocytochemistry and Immunohistochemistry. Third Conference of the European Society for Analytical Cellular Pathology, 1994; and AutoCyte Quic Immuno User's Manual, 1998, document number PA-029, Co AutoCyte Inc. Burlington NC 2721). The histograms and the score sheet indicated that each oligo were equally efficient in yielding a colorimetric signal. This indicates that the position of the label domain can be either 3 prime or 5 prime to the gene target sequence or the gene target sequence can be positioned between two label domains.

#### EXAMPLE 4

##### In Situ Hybridization

The probe collections prepared in Example 1 were first diluted in a solution consisting of 20% dextran sulfate (wt/vol), 50% formamide (vol/vol), 2X SSC, 10 mM Tris-HCl, 5 mM EDTA, and 0.05% Brij-35, at a final pH of 7.3. Probe collections were then mixed with an equal volume of a solution consisting of 2X SSC and 0.05% Triton X-100.

WO 02/022874

PCT/US01/28014

Samples for ISH analysis were prepared by cutting formalin-fixed and paraffin-embedded cells or tissue samples into 4 µm sections and placing the sections onto a glass slide. Subsequent processing and ISH of samples was carried out in an automated device, such as the DISCOVERY™ Automated ISH/IHC Stainer (Ventana Medical Systems, Inc., Tucson, AZ) described in co-owned and co-pending U.S. Patent App. Serial Nos. 60/076,198 and 09/259,240, both incorporated herein by reference. . To remove paraffin from the samples, the slides were immersed in an aqueous solution, heated for approximately 20 minutes, and then rinsed. The automated deparaffinization procedure is more fully described in U.S. Serial No. 60/099,018, 09/259,240 both incorporated herein by reference. The samples were then treated with protease and the slides were heated to 85°C (for hybridization to RNA target genes) or 90-95°C (for hybridization to DNA target genes) for 4 to 10 minutes.

Hybridization reactions were typically performed in a hybridization buffer consisting of 10% dextran sulfate (wt/vol), 25% formamide (vol/vol), 2X SSC, 5 mM Tris, 2.5 mM EDTA, 0.025% Brij-35, 0.25% Triton X-100, and between 25 to 125 ng/mL of each individual probe molecule. ISH reactions were performed at between 37°C to 54°C. For ISH using the probe collections described in Example 1, hybridization reactions were optimally carried out for 1 hr at 47°C (except for the poly d(T) probe, wherein the hybridization reaction was optimally carried out at 37°C for 1 hr).

The hybridization of fluorescein-labeled probe molecules to a particular target gene in the sample was detected by using a sequential series of binding proteins, i.e., secondary antibody detection. However, it is equally possible to use detect detection when visualizing the bound probes. In secondary detection, first, an anti-fluorescein mouse monoclonal antibody directed against the fluorescein-labeled probe molecule was added to the sample. Next, a biotin-labeled polyclonal goat antibody directed against the mouse antibody was added to the sample. Finally, hybridization reactions were colorimetrically detected using a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate. This technique, termed "secondary antibody detection," is routine for one of skill in the art. Primary and secondary antibodies are available from numerous suppliers, including Ventana Medical Systems, Tucson, AZ, which are optimized for use on the Ventana autostaining systems (ES<sup>®</sup>, NexES<sup>®</sup>, DISCOVERY™, and BENCHMARK™).

WO 02/022874

PCT/US01/28014

Figures 2-21 illustrate the results obtained for *in situ* hybridization analysis of various cell lines or tissue samples using the probes disclosed and claimed herein having the structural motif illustrated in Figure 1 or probe collections consisting of such probes.

Figure 1 illustrates a generic probe structure of the two-domain probe design. This is the oligonucleotide design used for the probes in the gene specific cocktails described in the following examples. Each probe is composed of two domains: a 5' labeling domain and a 3' target gene target gene-specific domain. The labeling domain consists of this specific sequence (CTATTTT)<sub>n</sub>, wherein the cytosine nucleotide is a cytosine-hapten conjugate, the hapten being fluorescein in this embodiment. This illustration specifically shows nucleic acid sequences for the 301 (SEQ ID NO: 55) and 302 (SEQ ID NO: 56) probes, each of which possesses target gene gene-specific domains corresponding to human repetitive Alu sequences and labeling domains having a fluorescein hapten.

Figure 2 illustrates the results obtained for *in situ* hybridization (ISH) analysis of human skin tissue using a probe comprising the labeling domain (330 probe; SEQ ID NO: 58). The absence of a detectable signal indicates that the sequence formula, (CTATTTT)<sub>n</sub>, of the labeling domain common to the oligonucleotides used in these ISH examples is non-specific, and non-reactive in its ability to form Watson-Crick base pairing with human nucleic acid sequences because it does not hybridize.

Figure 3 illustrates the results obtained for ISH analysis of human skin tissue using a probe comprising the labeling domain and a poly d(T) target gene-specific domain (320 probe; SEQ ID NO: 57). The presence of a detectable signal localized to the cytoplasm indicates that this probe is capable of specifically hybridizing to polyadenylated region of messenger RNA.

Figures 4A-4B illustrate the results obtained for ISH analysis of human skin tissue using the 320 probe, wherein the tissue sample was not treated with ribonuclease A prior to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The decrease in detectable signal in (B) indicates that this probe specifically hybridizes to polyadenylated region common to messenger RNA.

Figures 5A-5B illustrate the results obtained for ISH analysis of human spleen tissue using the 320 probe, wherein the hybridization and stringency wash were performed at room temperature (A), or at 37°C (B). This result illustrates that the

WO 02/022874

PCT/US01/28014

intensity of color is related to the stringency of hybridization conditions, with the more intense color indicating less stringent conditions.

Figure 6 illustrates the results obtained for ISH analysis of the human Raji cell line using the 320 probe. This shows that this probe design also is functional with embedded cell lines as well as embedded tissue.

Figure 7 illustrates the results obtained for ISH analysis of the human Raji cell line using a probe collection consisting of the 301 and 302 probes.

Figure 8 illustrates the results obtained for ISH analysis of the human HT cell line using a probe collection consisting of the 301 and 302 probes.

Figure 9 illustrates the results obtained for ISH analysis of a rat cell line using a probe collection consisting of the 301 and 302 probes. The absence of a detectable signal indicates that this probe collection is specific for human nucleic acid sequences.

Figure 10 illustrates the results obtained for ISH analysis of an Epstein-Barr virus (EBV)-negative human HT cell line using a probe possessing a target gene-specific domain corresponding to EBV EBER nuclear RNA [SEQ ID NO: 51 through SEQ ID NO: 54].

Figure 11 illustrates the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54].

Figure 12 illustrates the results obtained for ISH analysis of human tonsil tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54].

Figures 13A-13B illustrate the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54], wherein the tissue sample was not treated with ribonuclease A prior to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The decrease in detectable signal in (B) indicates that this probe specifically hybridizes to human EBER 1 and EBER 2 nuclear RNA.

WO 02/022874

PCT/US01/28014

Figure 14 illustrates the results obtained for ISH analysis of kappa light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NO:15].

Figure 15 illustrates the results obtained for ISH analysis of lymphoma tissues using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin kappa light chain mRNA [SEQ ID NOS: 2 - 4, SEQ ID NOS:7-12, SEQ ID NOS: 14, 15]. The lymphoma tissue in (A) over expresses the kappa light chain and the tissue in (B) over expresses the lambda light chain. The absence of a detectable signal in (B) indicates that the kappa light chain probe collection is specific to kappa light chain mRNA.

Figure 16 illustrates the results obtained for ISH analysis of lambda light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain variable region mRNA [SEQ ID NOS:19 through 29].

Figure 17 illustrates the results obtained for ISH analysis of a lambda light chain-positive human RPMI 8226 cell line using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NOS:19 through 29].

Figures 18A-18B illustrate the results obtained for ISH analysis of human lymphoma tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NOS:19 through 29]. The tissue in (A) over expresses the lambda light chain and the tissue in (B) over expresses the kappa light chain. The absence of a detectable signal in (B) indicates that the lambda light chain probe collection is specific to human lambda light chain mRNA.

Figure 19 illustrates the results obtained for ISH analysis of cytomegalovirus (CMV)-positive human lung tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA [SEQ ID NOS:30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50]. Arrow indicates CMV infected cell.

Figure 20 illustrates the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has not been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-

WO 02/022874

PCT/US01/28014

specific domains corresponding to CMV immediate early RNA [SEQ ID NOS:30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50].

Figures 21A-21B illustrate the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA [SEQ ID NOS:30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50] expression of the CMV immediate early RNA with cyclohexamide. The tissue in (A) is shown at a magnification of 40X and the tissue in (B) is shown at a magnification of 20X.

TABLE 1

Probe ID	Sequence	SEQ ID
401	5'-CTATTTCTATTTCTATTTCTATTTCT CCGAGTAGCAGGAGCCCCAGGAGCTGAGC-3'	1
402	5'-CTATTTCTATTTCTATTTCTATTTCT GGTGGAGACTGGGTCACTGGATGTCACA-3'	2
403	5'-CTATTTCTATTTCTATTTCTATTTCT GCAAGCGATGGTGACTCTGTCTCCFACAGC-3'	3
404	5'-CTATTTCTATTTCTATTTCTATTTCT TCTGTCCAGATCCACTGCCACTGAACCTT-3'	4
405	5'-CTATTTCTATTTCTATTTCTATTTCT GCAGCCACAGTTCGCTTCATCTGCACCTTG-3'	5
406	5'-CTATTTCTATTTCTATTTCTATTTCT TTTCRACTGCTCATCAGATGGCGGAGAT-3'	6
407	5'-CTATTTCTATTTCTATTTCTATTTCT AAGTTATTCAGCAGGCACACAGAGGCA-3'	7
408	5'-CTATTTCTATTTCTATTTCTATTTCT GGCCTTATCCACTTCCACTGTACTTTGGC-3'	8
409	5'-CTATTTCTATTTCTATTTCTATTTCT TAGGTGCTGTCTTGGCTCTCTGCTG-3'	9
410	5'-CTATTTCTATTTCTATTTCTATTTCT GTAGTCTGCTTTGCTCAGGCTCAGGGTGT-3'	10
411	5'-CTATTTCTATTTCTATTTCTATTTCT GATGGGTGACTTCGCAAGCGTACACTTTGT-3'	11
412	5'-CTATTTCTATTTCTATTTCTATTTCT CTCFCCCCTGTGAACTCTTTGTGACGG-3'	12
413	5'-CTATTTCTATTTCTATTTCTATTTCT TGGAACTGAGGAGCAGGTGGGGCACTTCT-3'	13
414	5'-CTATTTCTATTTCTATTTCTATTTCT GAAAAAGCGTCCAGAGGCCAAGGATGGAG-3'	14
415	5'-CTATTTCTATTTCTATTTCTATTTCT AGATGAGCTGGAGGACCCCAATAGGGGTAG-3'	15
416	5'-CTATTTCTATTTCTATTTCTATTTCT GCATAATTAAAGCCAAAGGAGGAGGGGG-3'	16
501	5'-CTATTTCTATTTCTATTTCTATTTCT CCTGAGTGAGGAGGGTGGAGCCAGCAGAG-3'	17
502	5'-CTATTTCTATTTCTATTTCTATTTCT AGACCCAGACACGGAGGCGAGCTGAGTCAG-3'	18
503	5'-CTATTTCTATTTCTATTTCTATTTCT TGTGGTCCAGTGCAGGAGATGCTGATCG-3'	19
504	5'-CTATTTCTATTTCTATTTCTATTTCT TAAATCATGATTTGGGGGCTTTGCCCTGGG-3'	20

- 17 -

SUBSTITUTE SHEET (RULE 26)

WO 02/022874

PCT/US01/28014

505	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>TGTGCCAGACTTGGAGCCAGAGAAGCGAT</u> -3'	21
506	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>AATAATCAGCCTCGTCTCAGCCTGGAGCC</u> -3'	22
507	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>GGTCTCCGCGGAAACCACAGGTGTAAC</u> -3'	23
508	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>TTATGAGACACACAGCTGGCCCTTGTG</u> -3'	24
509	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>CTGCTCAGGOSTCAGGCTCAGATAGCTGCT</u> -3'	25
511	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>ATGCGTGAACCTGGCAGCTGTAGCTCTG</u> -3'	26
512	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>ATTCGTAGGGCCACTGCTCTCTCCACGG</u> -3'	27
513	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>CCTCCCTGAGGATCCTGCAGCTCTAGTCTC</u> -3'	28
515	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>TGAGGGTTTATGAGTCCAGGGAGAAGGGC</u> -3'	29
221	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>GGAGTCAAAACAGCCTGGATGGCG</u> -3'	30
222	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>GAGGCTGGATCGGTCCGGTGTCTT</u> -3'	31
223	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>AATCCGCTTCCAAATGCAACCGTCC</u> -3'	32
224	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>TAAABAACTCGGSCACTGGGACGG</u> -3'	33
225	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>ACCGAGATTGCGTGGAGATCCCA</u> -3'	34
226	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>GAGCAAGGAGCTCCGACCGACCAT</u> -3'	35
227	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>ACACTGGTGGTGGTGGCCATCGTGC</u> -3'	36
228	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>TTCCAAATGCGTCAGCGGTGCAAGC</u> -3'	37
229	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>AGCTGCTGCATCTTCTTCTGCCGC</u> -3'	38
230	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>CCCTCCACCCTTAAACAGCACCCAA</u> -3'	39
231	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>TTGGTCCAGGGTCTCTGGGSCCTAA</u> -3'	40
232	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>TCGGCCAACTCTGAAACAGCGGAT</u> -3'	41
233	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>TCGGGGTCTCGTTCGCAATCCTCGG</u> -3'	42
234	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>ATCTGGATGCCCCGCTCACATGCAA</u> -3'	43
235	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>TGCCGCACCATGCTCACTCGAACCT</u> -3'	44
236	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>GTTAGCGGGCCCTTGTCCACATCA</u> -3'	45
237	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>TGCAGATCTCCTCAATCGCGGCTT</u> -3'	46
238	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>TCTCAGAGGATGGCCCCAGATG</u> -3'	47
239	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>CCTCACTGACTCCCGGGATGGC</u> -3'	48
240	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>CGGGTACAGGGGACTCTGGGGTGA</u> -3'	49
241	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>GGGTGGTCTCTTGCCTCCAGAGG</u> -3'	50
100A2	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>GACCTCGGGTGGTATGACACCGCACT</u> -3'	51
100C2	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>GGAAAGCTCTCTCTCTCCGCCG</u> -3'	52
100A1	5'-CTATTTTCATTTTCATTTTCATTTTC	<u>CCACAGACACCGTCCCTCACCACCCG</u> -3'	53

WO 02/022874

PCT/US01/28014

100B1	5'-CTATTTTCATTTTCATTTTCATTTTC	GGCACAGCCACACACGTCCTCCCTCC-3'	54
301	5'-CTATTTTCATTTTCATTTTCATTTTC	CGAGGCGGGCGGATCACCTGAGGTC-3'	55
302	5'-CTATTTTCATTTTCATTTTCATTTTC	CGGAGGCGGAGGTTGCAGTGAGCC-3'	56
320	5'-CTATTTTCATTTTCATTTTCATTTTC	TTTTTTTTTTTTTTTTTTTTTTTT-3'	57
301A	5'-CTATTTTCATTTTCATTTTCATTTTC	CGAGGCGGGCGGATCACCTGAGGTC-3'	116
302C	5'-CTATTTTCATTTTCATTTTCATTTTC	CGGAGGCGGAGGTTGCAGTGAGCC-3'	117
302A4	5'-CTATTTTACTTTTATATTTTCT	CGGAGGCGGAGGTTGCAGTGAGCC-3'	118
	5'-CTATTTTATATTTTCT	CGGAGGCGGAGGTTGCAGTGAGCC ACTATTTTACTTT-3	119
302A3 /2			
1002A 32	5'-CTATTTTACTTTTATATTTTCT	GACCTCGGTCGGTAGCACCACCAC TACTATTTTACTTT-3'	120
301A 2/2	5'-CTATTTTCTT	CGAGGCGGGCGGATCACCTGAGGTC TTCTTTTATCTT-3	121
301A 3/2	5'-CTATTTTACTTTTATATTTTCT	CGAGGCGGGCGGATCACCTGAGGTC ACTATTTTACTTT-3'	122

Table 2

Probe ID	Sequence	SEQ ID
	5'-CTATTTTCATTTTCATTTTCATTTTC	123
	5'-CTATTTTACTTTTATATTTTCT	124
330	5'-CTATTTTCATTTTCATTTTCATTTTC	58
	5'-CTATTTTACTTTTATATTTTCT.....ACTATTTTACTTT-3	125
	5'-CTATTTTCTT.....TTCTTTTATCTT-3	126

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

WO 02/022874

PCT/US01/28014

## WE CLAIM:

1. An oligonucleotide label-domain comprising the sequence  $(CTATTTT)_n$  and its complement  $(AAAATAG)_n$ , wherein "n" is at least 1.
2. The oligonucleotide label-domain of claim 1 detectably labeled with a reporter molecule, or a hapten molecule.
3. The oligonucleotide label-domain of claim 2 wherein the hapten is flurorescein linked to the N4 nitrogen of cytosine through an OB EA linker.
4. The oligonucleotide label-domain of claim 1 wherein the reporter molecule is a fluorophore.
5. The oligonucleotide label-domain of claim 1 wherein the fluorophore is present at a density of greater than 7 mole percent.
6. The oligonucleotide label-domain of claim 1 wherein the label-domain has the sequence  $TC(TTTATC)_n$  ( or its complementary formula).
7. The oligonucleotide label-domain of claim 1 wherein the sequence is SEQ ID NO: 58.
8. The oligonucleotide label-domain of claim 2 wherein at least 7 mole percent of the cytosines are linked to a detectable moiety by an OB EA linker.

WO 02/022874

PCT/US01/28014

9. An oligonucleotide probe having at least two distinct functional domains, a first domain comprising the label-domain of claim 2, and a second domain comprising a gene-specific target sequence.
10. The oligonucleotide probe of claim 9 wherein the label-domain is located at the 5' end of the oligonucleotide probe, and the gene-specific target sequence being 3' to the label-domain.
11. The oligonucleotide probe of claim 9 wherein the label-domain is located at the 3' end of the oligonucleotide probe, and the gene-specific target sequence is 5' to the label-domain.
12. An oligonucleotide probe having three distinct functional domains, a first domain comprising the label-domain of claim 2, a second domain comprising a gene-specific target sequence, and a third domain comprising another label-domain, wherein said second domain is located between said first and third domains.
13. A probeset for detecting Kappa immunoglobulin light chain mRNA or corresponding heteronuclear RNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 401 through 416, inclusive.
14. A probeset for detecting Lambda immunoglobulin light chain mRNA or corresponding heteronuclear RNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 501 through 509, 511-513, and 515.

WO 02/022874

PCT/US01/28014

15. A probeset for detecting cytomegalovirus (CMV) immediate early RNA and /or corresponding mRNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 221 through 241.
  
16. A probeset for detecting Epstein Barr virus (EBV) early RNA, RNA 1 and RNA 2, (EBER) wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 51 through 54.
  
17. A probeset for detecting Human Alu repetitive satellite genomic DNA sequences wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 301 and 302.

# Oligonucleotide Probe Design

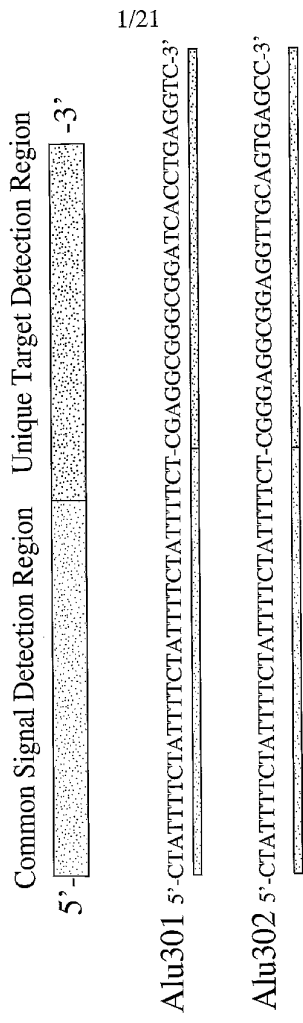


FIG. 1

WO 02/022874

PCT/US01/28014

2/21



FIG. 2

SUBSTITUTE SHEET (RULE 26)

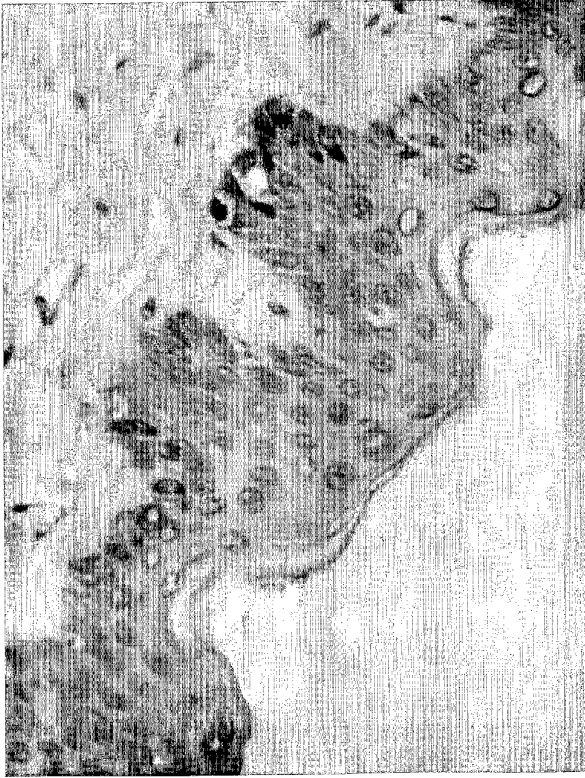


FIG. 3

PLUS RNase



FIG. 4B

MINUS RNase



FIG. 4A

WO 02/022874

PCT/US01/28014

5/21

37/37

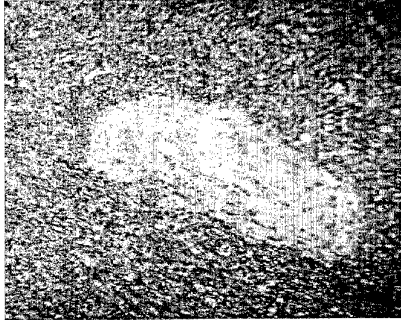


FIG. 5B

RT/RT

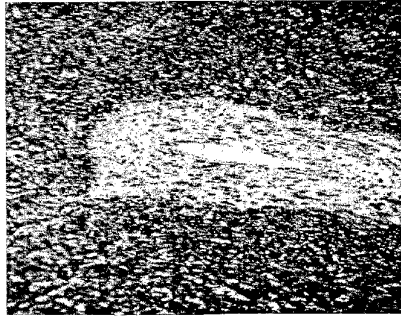


FIG. 5A

SUBSTITUTE SHEET (RULE 26)

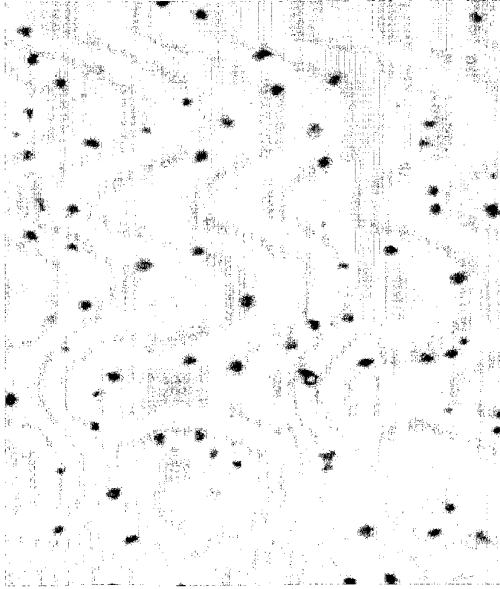


FIG. 6

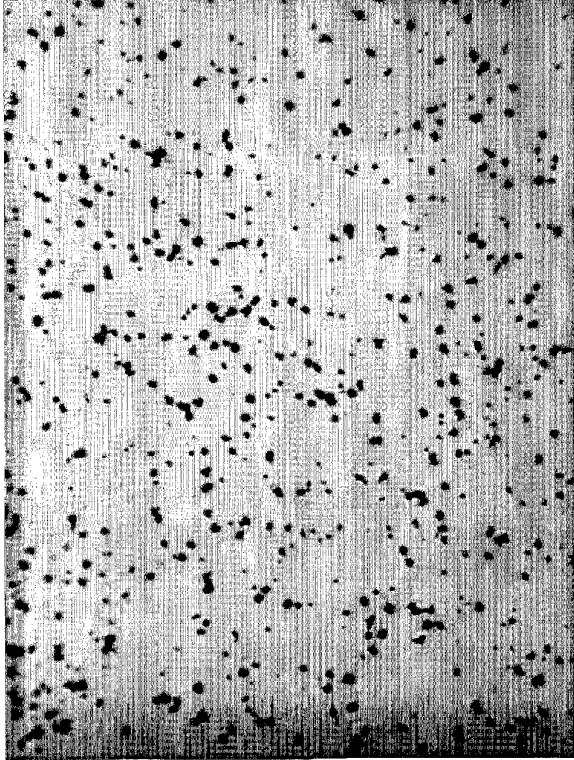


FIG. 7

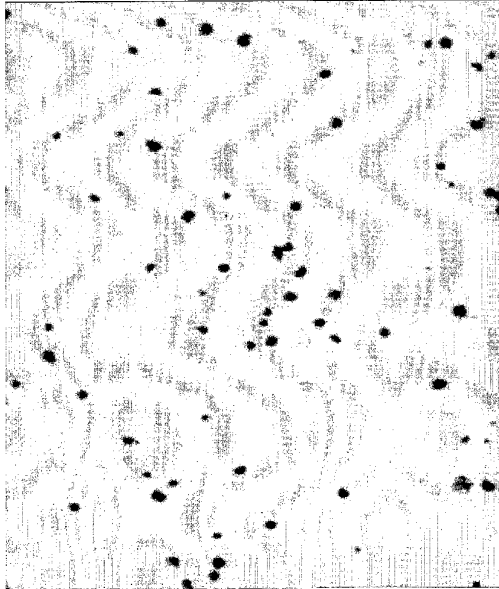


FIG. 8

WO 02/022874

PCT/US01/28014

9/21

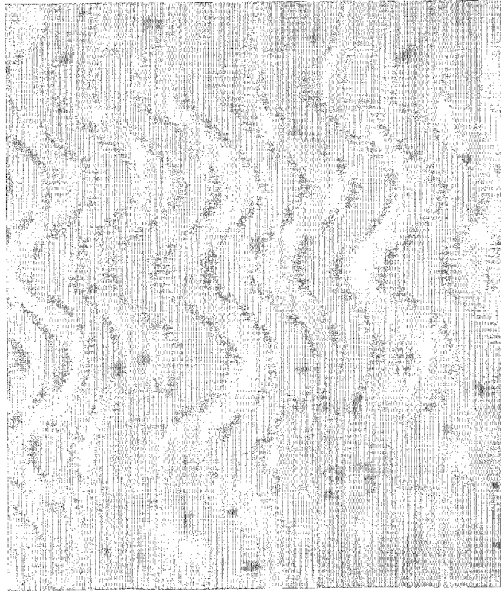


FIG. 9

SUBSTITUTE SHEET (RULE 26)

WO 02/022874

PCT/US01/28014

11/21

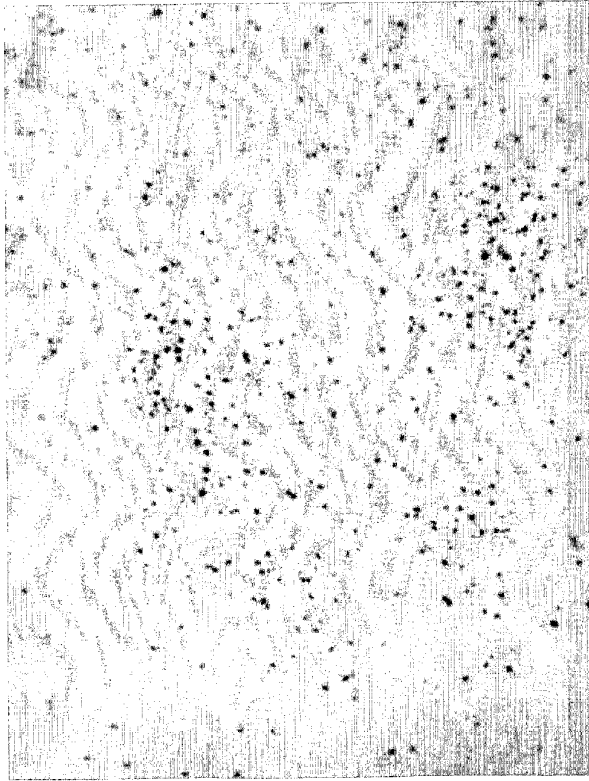


FIG. 11

SUBSTITUTE SHEET (RULE 26)

WO 02/022874

PCT/US01/28014

12/21

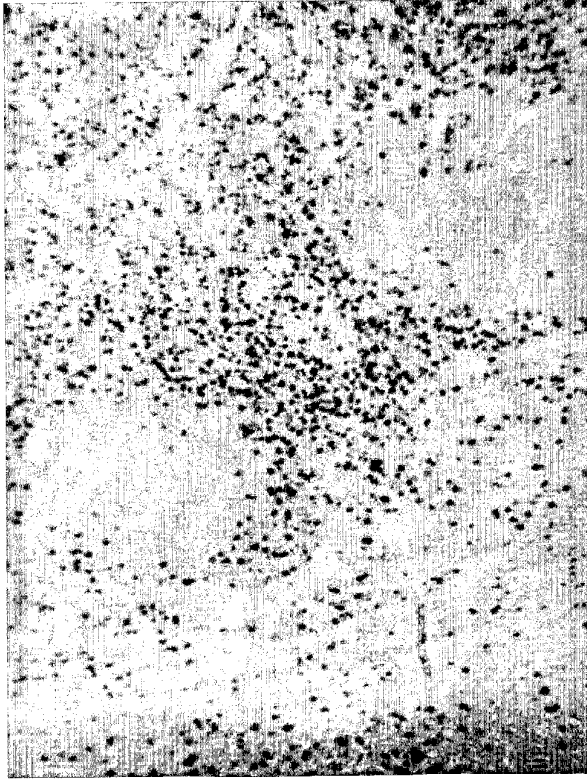


FIG. 12

SUBSTITUTE SHEET (RULE 26)

PLUS RNase



FIG. 13B

MINUS RNase



FIG. 13A

WO 02/022874

PCT/US01/28014

10/21

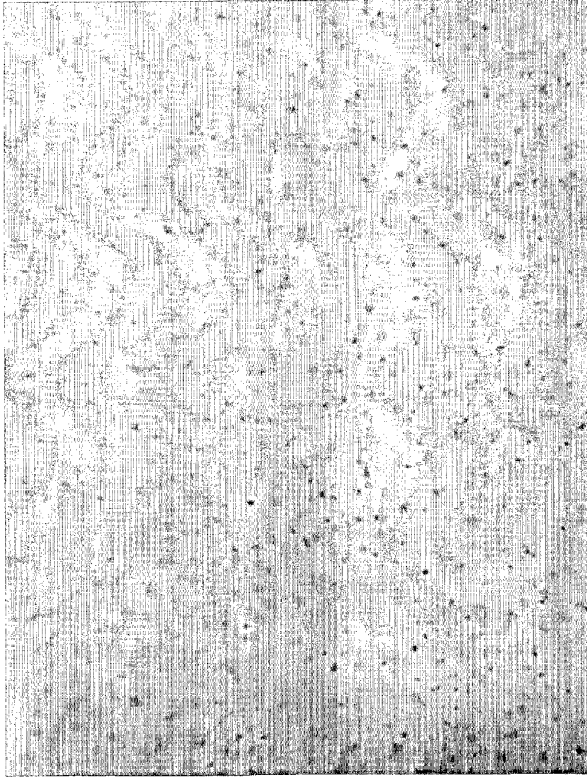


FIG. 10

SUBSTITUTE SHEET (RULE 26)

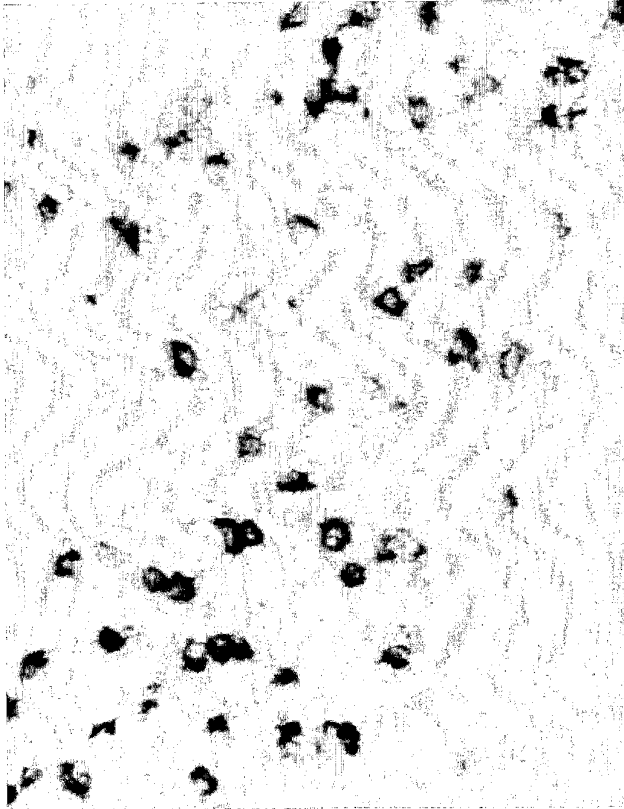


FIG. 14



FIG. 15B



FIG. 15A

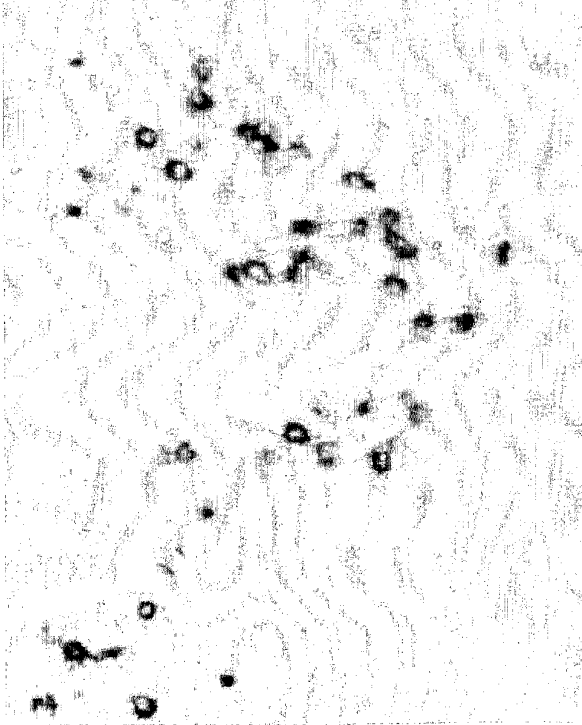


FIG. 16

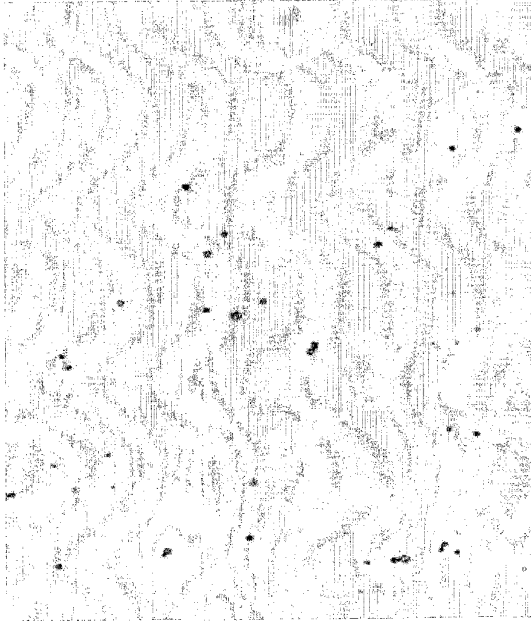


FIG. 17



FIG. 18B



FIG. 18A

WO 02/022874

PCT/US01/28014

19/21

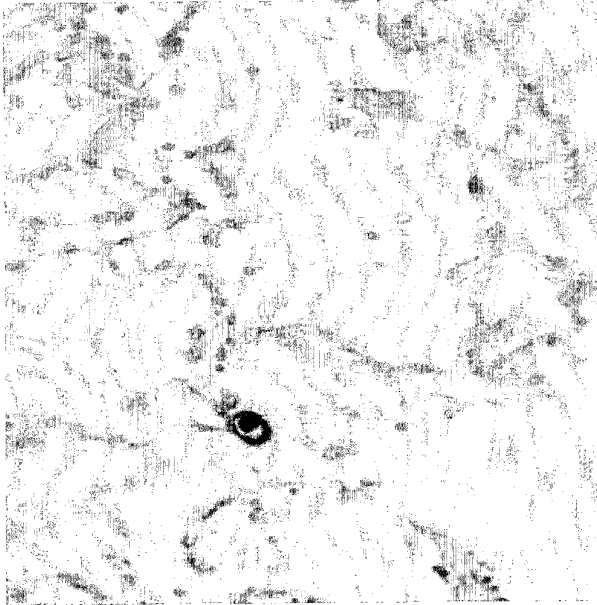


FIG. 19

SUBSTITUTE SHEET (RULE 26)

WO 02/022874

PCT/US01/28014

20/21



FIG. 20

SUBSTITUTE SHEET (RULE 26)

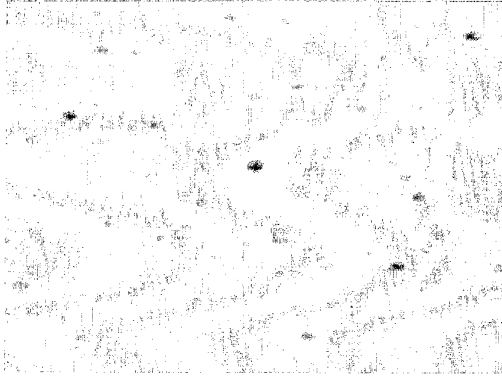


FIG. 21B

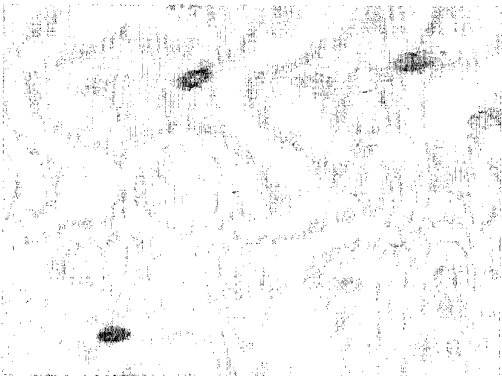


FIG. 21A

WO 02/022874

PCT/US01/28014

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<110> Utermohlen, Joseph  
<110> Connaughton, John

<120> Oligonucleotide sequence formula for labeling Oligonucleotide Probes and Proteins  
for *In Situ* Analysis

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WO 02/022874

PCT/US01/28014

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WO 02/022874

PCT/US01/28014

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WO 02/022874

PCT/US01/28014

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WO 02/022874

PCT/US01/28014

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WO 02/022874

PCT/US01/28014

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WO 02/022874

PCT/US01/28014

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WO 02/022874

PCT/US01/28014

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WO 02/022874

PCT/US01/28014

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Oligonucleotide probe

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Oligonucleotide probe

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<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 41  
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<210> 42

WO 02/22874

PCT/US01/28014

<211> 55  
<212> DNA  
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<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 42  
ctattttcta ttttctatct tctattttct tgggggtct cgttgaatc ctggg 55

<210> 43  
<211> 55  
<212> DNA  
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<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 43  
ctattttcta ttttctatct tctattttct atctcgatgc cccgctcaaa tgcaa 55

<210> 44  
<211> 55  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 44  
ctattttcta ttttctatct tctattttct tgccgcacca tgtccactcg aacct 55

<210> 45  
<211> 55  
<212> DNA  
<213> Artificial Sequence

<220>  
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Oligonucleotide probe

<400> 45  
ctattttcta ttttctatct tctattttct gttagcggcg ccttgctca catca 55

<210> 46  
<211> 55  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 46

WO 02/022874

PCT/US01/28014

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ctattttcta tttctatatt tctattttct tgcagatccc ctcaatggcg egctt      55
<210> 47
<211> 55
<212> DNA
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<223> Description of Artificial Sequence:
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<400> 47
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<210> 48
<211> 55
<212> DNA
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<223> Description of Artificial Sequence:
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<400> 48
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<210> 49
<211> 55
<212> DNA
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ctattttcta tttctatatt tctattttct cgggtacagg ggactctggg ggtga      55
<210> 50
<211> 55
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<211> 55
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<220>
<223> Description of Artificial Sequence:

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WO 02/022874

PCT/US01/28014

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<210> 52  
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<400> 52  
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<210> 53  
<211> 55  
<212> DNA  
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<220>  
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Oligonucleotide probe

<400> 53  
ctatatttcta ttttctatatt tctatatttct ccacagacac cgtctcacc acccg 55

<210> 54  
<211> 56  
<212> DNA  
<213> Artificial Sequence

<220>  
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Oligonucleotide probe

<400> 54  
ctatatttcta ttttctatatt tctatatttct ggetacagcc acacacgtct cctccc 56

<210> 55  
<211> 55  
<212> DNA  
<213> Artificial Sequence

<220>  
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Oligonucleotide probe

<400> 55  
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<210> 56  
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WO 02/022874

PCT/US01/28014

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<220>
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<400> 56
ctatatttcta tttctattt tctatatttct cgggagggcgg aggttgccagt gagcc      55

<210> 57
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 57
ctatatttcta tttctattt tctatatttct tttttttttt tttttttttt tttttttttt 60

<210> 58
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 58
ctatatttcta tttctattt tctatatttct                                30

<210> 59
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 59
ccagagttagc aggagcccca ggagctgagc                                30

<210> 60
<211> 30
<212> DNA
<213> Artificial Sequence

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<223> Description of Artificial Sequence:
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<400> 60
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<210> 61

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WO 02/022874

PCT/US01/28014

<211> 30  
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<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 61  
gcaagcgatg gtgactctgt ctctacagc 30

<210> 62  
<211> 30  
<212> DNA  
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<220>  
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Oligonucleotide probe

<400> 62  
tctgtcccag atccactgcc actgaacctt 30

<210> 63  
<211> 30  
<212> DNA  
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<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 63  
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<210> 64  
<211> 30  
<212> DNA  
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<220>  
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Oligonucleotide probe

<400> 64  
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<210> 65  
<211> 30  
<212> DNA  
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<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 65

WO 02/022874

PCT/US01/28014

aagttattca gcaggcacac aacagaggca 30  
<210> 66  
<211> 30  
<212> DNA  
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<210> 67  
<211> 30  
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tagtgctgt cttgtgtc ctgctctgtg 30  
<210> 68  
<211> 30  
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glagctgct ttgetcagcg tcagggtgct 30  
<210> 69  
<211> 30  
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gatgggtgac ttgcagcg tagactttgt 30  
<210> 70  
<211> 30  
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WO 02/022874

PCT/US01/28014

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 <211> 30  
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 <210> 72  
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 <210> 73  
 <211> 30  
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 <210> 74  
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 <400> 74  
 gcataattaa agccaaggag gaggaggggg 30  
  
 <210> 75  
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WO 02/022874

PCT/US01/28014

<220>  
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<400> 75  
 cctgagtgag gagggtgagg agcagcagag 30

<210> 76  
 <211> 30  
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<220>  
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<400> 76  
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<210> 77  
 <211> 30  
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<220>  
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<400> 77  
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<210> 78  
 <211> 30  
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<220>  
 <223> Description of Artificial Sequence:  
 Oligonucleotide probe

<400> 78  
 taaatcatga ttttgggggc ttgcctggg 30

<210> 79  
 <211> 30  
 <212> DNA  
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<220>  
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 Oligonucleotide probe

<400> 79  
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<210> 80

WO 02/022874

PCT/US01/28014

<211> 30  
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 <223> Description of Artificial Sequence:  
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 <210> 81  
 <211> 30  
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 <223> Description of Artificial Sequence:  
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 <400> 81  
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 <210> 82  
 <211> 30  
 <212> DNA  
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 <223> Description of Artificial Sequence:  
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 <400> 82  
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 <210> 83  
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 <223> Description of Artificial Sequence:  
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 <400> 83  
 ctgctcaggc gtcaggctca gatagctgct 30  
  
 <210> 84  
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 <223> Description of Artificial Sequence:  
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 <400> 84

WO 02/022874

PCT/US01/28014

atgcgtgacc tggcagctgt agcttctgtg 30

<210> 85  
<211> 30  
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<400> 85  
attctgtagg ggccaactgtc ttctccacgg 30

<210> 86  
<211> 30  
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<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 86  
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<210> 87  
<211> 30  
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<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 87  
tgagggttta ttgagtcag ggagaagggc 30

<210> 88  
<211> 25  
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<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 88  
ggaggtcaaa acagcgtgga tggcg 25

<210> 89  
<211> 25  
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<220>  
<223> Description of Artificial Sequence:

WO 02/022874

PCT/US01/28014

## Oligonucleotide probe

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 gagcctggat cggteccggg gtctt 25  
  
 <210> 90  
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 taaaaactgc gggcaotggg gacgg 25  
  
 <210> 92  
 <211> 25  
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 acccgagatt cggctggaga tccca 25  
  
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WO 02/022874

PCT/US01/28014

<220>  
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Oligonucleotide probe

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acaactgggtgg tgggtgggcat cgtgc 25

<210> 95  
<211> 25  
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<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 95  
ttccaaatgc gtcagcggtg caagc 25

<210> 96  
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<400> 96  
agctgctgtgc atcttcttct gccgc 25

<210> 97  
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Oligonucleotide probe

<400> 97  
cctctcaccg ttaacagcac cgcaa 25

<210> 98  
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<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 98  
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<210> 99

WO 02/022874

PCT/US01/28014

<211> 25  
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<210> 101  
<211> 25  
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atctcgatgc ccgctcaca tgcaa 25  
  
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WO 02/022874

PCT/US01/28014

glttagcggcg cccttgetca catca 25

<210> 104  
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<400> 104 25  
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<210> 105  
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<400> 105 25  
 tctcagagga tggccccca gaatg

<210> 106  
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<400> 106 25  
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<210> 107  
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<400> 107 25  
 cgggtacagg ggactctggg ggtga

<210> 108  
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<220>  
 <223> Description of Artificial Sequence:

WO 02/022874

PCT/US01/28014

Oligonucleotide probe

<400> 108  
gggtgggtgc tttgcctcc agagg 25

<210> 109  
<211> 25  
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Oligonucleotide probe

<400> 109  
gacctcgggt cggtagcacc gcaat 25

<210> 110  
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<220>  
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Oligonucleotide probe

<400> 110  
ggaagcctct cttctctccc cccgg 25

<210> 111  
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<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 111  
ccacagacac cgtctcacc acccg 25

<210> 112  
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Oligonucleotide probe

<400> 112  
ggctacagcc acacagctc cctccc 26

<210> 113  
<211> 25  
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WO 02/022874

PCT/US01/28014

<220>  
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cgaggcgggc ggatcacctg aggtc 25

<210> 114  
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<220>  
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Oligonucleotide probe

<400> 114  
cgggaggcgg aggttgcaat gagcc 25

<210> 115  
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<212> DNA  
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<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 115  
tttttttttt tttttttttt tttttttttt 30

<210> 116  
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<212> DNA  
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<220>  
<223> Description of Artificial Sequence:  
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<400> 116  
CTATTTTCTATTTTCTTTT CGAGGCGGGGGATCACCTGAGGTC 46

<210> 117  
<211>  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 117  
CTATTTTCTATTTTCTTTT CGGAGCGGGAGGTTCAGTGAACC 46

<210> 118

WO 02/022874

PCT/US01/28014

<211>  
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<223> Description of Artificial Sequence:  
Oligonucleotide probe  
  
<400> 118  
CTATTTTATACTTTATATTTTCATATTTTATCT CCGGAGCGCGAGGTTGCAGTGAGCC 57  
  
<210> 119  
<211>  
<212> DNA  
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<223> Description of Artificial Sequence:  
Oligonucleotide probe  
  
<400> 119  
CTATTTTATACTTTATATTTTCT CCGGAGCGCGAGGTTGCAGTGAGCC ACTATTTTATACTT 61  
  
<210> 120  
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<212> DNA  
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<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe  
  
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CTATTTTATACTTTATATTTTCT GACCTCGGGTCGGTAGCACCGGCAC TACTATTTTATACTT 62  
  
<210> 121  
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<210> 122  
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<212> DNA  
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Oligonucleotide probe

WO 02/022874

PCT/US01/28014

<400> 122  
CTATTTTATACTTTTATATTTCT CGAGGGGGGGGGATCACCTGAGGTC ACTATTTTATACTT  
61

<210> 123  
<211>  
<212> DNA  
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<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 123  
CTATTTTCTATTTTCTTTT 21

<210> 124  
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<223> Description of Artificial Sequence:  
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<400> 124  
CTATTTTATACTTTATATTTTCATATTTTACT 32

<210> 125  
<211>  
<212> DNA  
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<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 125  
CTATTTTATACTTTTATATTTCTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNACTATTTTATACTT 61

<210> 126  
<211>  
<212> DNA  
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<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 126  
CTATTTTCTTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTTCTTTTATCTT 49

## 【国際公開パンフレット(コレクション)】

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
21 March 2002 (21.03.2002)

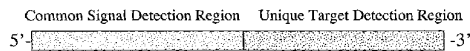
PCT

(10) International Publication Number  
WO 02/022874 A2

- (51) International Patent Classification: C12Q 1/68 85711 (US), CONNAUGHTON, John [US/US]; 9117 Hummester Road, Laytonsville, MD 20882 (US).
- (21) International Application Number: PCT/US01/28014
- (22) International Filing Date: 6 September 2001 (06.09.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 60/233,177 15 September 2000 (15.09.2000) US
- (71) Applicant (for all designated States except US): VENTANA MEDICAL SYSTEMS, INC. [US/US]; 3865 N. Business Center Drive, Tucson, AZ 85705 (US).
- (72) Inventors; and  
(75) Inventors/Applicants (for US only): UTERMOHLEN, Joseph, G. [US/US]; 5357 East 19th Street, Tucson, AZ
- (74) Agent: JONES, Huw, R.; Ventana Medical Systems, Inc., 1910 Innovation Park Drive, Tucson, AZ 85737 (US).
- (81) Designated States (national): AU, CA, JP, MX, US.
- (84) Designated States (regional): European patent (AT, BE, CH, CY, DE, DK, ES, FR, GB, GR, IT, LI, LU, MC, NL, PT, SE, TR).
- (48) Date of publication of this corrected version: 10 July 2003
- (15) Information about Corrections: see PCT Gazette No. 28/2003 of 10 July 2003, Section II
- [Continued on next page]*

(54) Title: OLIGONUCLEOTIDE SEQUENCE FORMULA FOR LABELING OLIGONUCLEOTIDE PROBES AND PROTEINS FOR *IN SITU* ANALYSIS

## Oligonucleotide Probe Design



Alu301 5'-CTATTTCTATTTCTATTTCTATTTCT-CGAGCGGGCGGATCACCTGAGTC-3'

Alu302 5'-CTATTTCTATTTCTATTTCTATTTCT-CGGGAGGCGGAGGTTGCAGTGAGCC-3'

(57) Abstract: The present invention provides oligonucleotide probes and oligonucleotide probe collections and protein labeling for detecting or localizing a plurality nucleic acid target genes or antigens within a cell or tissue sample. Specifically, the provides collections of oligonucleotide probe for use in *in situ* hybridization analyses in which each probe has a label-domain with the sequence formulas of (CTATTT)<sub>n</sub>? (AAAATAG)<sub>n</sub>? or (TTTTATC)<sub>n</sub>? or (GATAAAA)<sub>n</sub>? in which all cases "n" would equal 1 or greater. The present invention provides collections or "cocktails" of oligonucleotide probes for detecting or localizing specific nucleic acid target genes within a cell or tissue sample. The cocktails are useful for detecting the following: the Kappa gene (SEQ ID NOS: 1-16 inclusive); the Lambda gene (SEQ ID NOS: 501-509, 511-513, and 515); the CMV (cytomegalovirus) gene (SEQ ID NOS: 221-241 inclusive); EBV (Epstein-Barr RNA) gene (SEQ IN NOS: 51-54 inclusive); Alu (SEQ IN NOS: 55-56); PolyA (SEQ ID NO: 57); and the detection tail (SEQ ID NO: 330).

WO 02/022874 A2

**WO 02/022874 A2** 

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**Previous Correction:**  
see PCT Gazette No. 14/2003 of 3 April 2003, Section II

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

## 【国際公開パンフレット(コレクション)】

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
21 March 2002 (21.03.2002)

PCT


(10) International Publication Number  
WO 02/022874 A3

- (51) International Patent Classification: C12Q 1/68 (74) Agent: JONES, Huw, R.: Ventana Medical Systems, Inc., 1910 Innovation Park Drive, Tucson, AZ 85737 (US).
- (21) International Application Number: PCT/US01/28014 (81) Designated States (national): AU, CA, JP, MX, US.
- (22) International Filing Date: 6 September 2001 (06.09.2001) (84) Designated States (regional): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).
- (25) Filing Language: English
- (26) Publication Language: English Published: with international search report
- (30) Priority Data: 60/233,177 15 September 2000 (15.09.2000) US (88) Date of publication of the international search report: 25 September 2003
- (71) Applicant (for all designated States except US): VENTANA MEDICAL SYSTEMS, INC. [US/US]; 3865 N. Business Center Drive, Tucson, AZ 85705 (US). (15) Information about Corrections:  
Previous Corrections:  
see PCT Gazette No. 28/2003 of 10 July 2003, Section II  
see PCT Gazette No. 14/2003 of 3 April 2003, Section II
- (72) Inventors: and  
(75) Inventors/Applicants (for US only): UTERMÖHLEN, Joseph, G. [US/US]; 5357 East 19th Street, Tucson, AZ 85711 (US). CONNAUGHTON, John [US/US]; 9117 Huntmaster Road, Laytonsville, MD 20882 (US).  
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: OLIGONUCLEOTIDES FOR LABELING OLIGONUCLEOTIDE PROBES AND PROTEINS

## Oligonucleotide Probe Design

Common Signal Detection Region Unique Target Detection Region

5'--3'

Alu301 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT-CGAGGGCGGGGATCACTGAGGTC-3'

Alu302 5'-CTATTTTCTATTTTCTATTTTCTATTTTCT-CGGGAGCGGGAGGTTGCAGTGAGCC-3'

(57) Abstract: The present invention provides oligonucleotide probes and oligonucleotide probe collections and protein labeling for detecting or localizing a plurality nucleic acid target genes or antigens within a cell or tissue sample. Specifically, the provides collections of oligonucleotide probe for use in *in situ* hybridization analyses in which each probe has a label-domain with the sequence formulas of (CTATTT)<sub>n</sub>, (AAAATAG)<sub>n</sub>, or (TTTATC)<sub>n</sub>, or (GATAAAA)<sub>n</sub>, in which all cases "n" would equal 1 or greater. The present invention provides collections or "cocktails" of oligonucleotide probes for detecting or localizing specific nucleic acid target genes within a cell or tissue sample. The cocktails are useful for detecting the following: the Kappa gene (SEQ ID NOS: 1-16 inclusive); the Lambda gene (SEQ ID NOS: 501-509, 511-513, and 515); the CMV (cytomegalovirus) gene (SEQ ID NOS: 221-241 inclusive); EBV (Epstein-Barr RNA) gene (SEQ IN NOS: 51-54 inclusive); Alu (SEQ IN NOS: 55-56); PolyA (SEQ ID NO: 57); and the detection tail (SEQ ID NO: 330).

WO 02/022874 A3

【国際公開パンフレット(コレクトバージョン)】

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
Internationale Bureau



(43) International Publication Date  
21 March 2002 (21.03.2002)

PCT

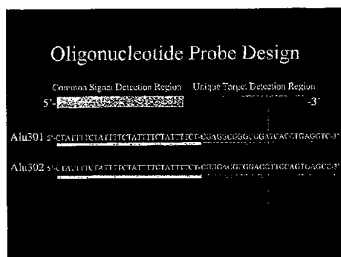
(10) International Publication Number  
WO 02/22874 A2

- (51) International Patent Classification: C12Q 1/68 Joseph, G. [US/US]; 5357 East 19th Street, Tucson, AZ 85711 (US). CONNAUGHTON, John [US/US]; 9117 Huntmaster Road, Laytonsville, MD 20882 (US).
- (21) International Application Number: PCT/US01/28014
- (22) International Filing Date: 6 September 2001 (06.09.2001) (74) Agent: JONES, Huw, R.; Ventana Medical Systems, Inc., 3865 N. Business Center Drive, Tucson, AZ 85705 (US).
- (25) Filing Language: English (81) Designated States (national): AU, CA, JP, MX, US.
- (26) Publication Language: English (84) Designated States (regional): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).
- (30) Priority Data: 60/233,177 15 September 2000 (15.09.2000) US Published: — without international search report and to be republished upon receipt of that report
- (71) Applicant (for all designated States except US): VENTANA MEDICAL SYSTEMS, INC. [US/US]; 3865 N. Business Center Drive, Tucson, AZ 85705 (US).

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

- (72) Inventors: and
- (75) Inventors/Applicants (for US only): UTERMOHLEN,

(54) Title: OLIGONUCLEOTIDE SEQUENCE FORMULA FOR LABELING OLIGONUCLEOTIDE PROBE AND PROTEINS FOR IN-SITU ANALYSIS



WO 02/22874 A2

(57) Abstract: The present invention provides oligonucleotide probes and oligonucleotide probe collections and protein labeling for detecting or localizing a plurality nucleic acid target genes or antigens within a cell or tissue sample. Specifically, the provides collections of oligonucleotide probe for use in *in situ* hybridization analyses in which each probe has a label-domain with the sequence formulas of (C1A1TTT)<sub>n</sub>, (AAAATAG)<sub>n</sub>, or (TTTATC)<sub>n</sub>, or (GATAAAA)<sub>n</sub>, in which all cases "n" would equal 1 or greater. The present invention provides collections or "cocktails" of oligonucleotide probes for detecting or localizing specific nucleic acid target genes within a cell or tissue sample. The cocktails are useful for detecting the following: the Kappa gene (SEQ ID NOS: 1-16 inclusive); the Lambda gene (SEQ ID NOS: 501-509, 511-513, and 515); the CMV (cytomegalovirus) gene (SEQ ID NOS: 221-241 inclusive); EBV (Epstein-Barr RNA) gene (SEQ IN NOS: 51-54 inclusive); A1u (SEQ IN NOS: 55-56); PolyA (SEQ ID NO: 57); and the detection tail (SEQ ID NO: 330).

## BACKGROUND OF THE INVENTION

1. **Field of the Invention**

This invention relates to oligonucleotide probes and collections of oligonucleotide probes for detecting or localizing nucleic acid genes targets within a cell or tissue sample. In particular, the invention relates to collections of oligoprobes.

2. **Background of the Invention**

*In situ* analysis includes *in situ* hybridization and immunohistochemistry. *In situ* hybridization (ISH) employs labeled DNA or RNA probe molecules that are anti-sense to a target gene sequence or transcript to detect or localize targeted nucleic acid target genes within a cell or tissue sample. ISH has proven to be a useful tool in a number of biomedical fields, including developmental biology, cell biology, and molecular biology. ISH has been used, for example, to diagnose genetic disorders, map genes, study gene expression, and localize sites of target gene expression.

Typically, ISH is performed by exposing a cell or tissue sample immobilized on a glass slide to a labeled nucleic acid probe which is capable of specifically hybridizing to a given target gene in the cell or tissue sample (*In Situ Hybridization: Medical Applications* (G. R. Coulton and J. de Belleruche, eds., Kluwer Academic Publishers, 1992); *In Situ Hybridization: In Neurobiology; Advances in Methodology* (J. H. Eberwine, K. L. Valentino, and J. D. Barchas, eds., Oxford University Press, 1994); *In Situ Hybridization: A Practical Approach* (D. G. Wilkinson, ed., Oxford University Press, 1992)). The hybridization of labeled probe molecules to nucleic acids in the cell or tissue sample can then be detected using, for example, radioactive-based direct detection methods, fluorescence-based direct detection methods, or indirect detection methods based on the binding of a fluorescence-labeled protein binding to a hapten such as BrdU, digoxigenin-labeled or biotin-labeled nucleotides incorporated into probes. Hapten-based methods have been further extended to include those molecules to be bonded by binding protein-enzyme conjugates such as antibody-enzyme-conjugates and colorimetric based detection chemistry. In addition, several target genes can be simultaneously analyzed by exposing a cell or tissue sample to a plurality of nucleic acid probes that have been labeled with a plurality of different nucleic

WO 02/22874

PCT/US01/28014

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acid tags. For example, a plurality of nucleic acid probes can be labeled with a plurality of fluorescent compounds having different emission wavelengths, thereby permitting simultaneous multicolored analysis to be performed in a single step on a single target cell or tissue sample.

5 A significant problem associated with incorporation of labeled nucleotides into oligonucleotide probes is that the conjugation moieties that are attached to the nucleotide usually interfere with the formation of Watson-Crick base pairing, thus negatively affecting the hybridization of the probe to its target. This has been seen with use of label attached via N4-substituted cytosine nucleotides, because of steric hindrance and the expected shift to the less reactive state of a secondary amine (as seen with N4 labeled cytosine), as compared to the natural G-C bond formed with an unsubstituted cytosine (a primary amine). Any small change or interference with G-C bonding in a small oligonucleotide (25 to 50 bases) can reduce the ability of these oligos to hybridize with the intended targeted sequence.

10 There remains a need in the art to develop suitable probe designs for incorporating labeled nucleotides in oligonucleotide probes. We demonstrate that a few artificial sequences are viable alternatives for probe labeling and also work both singly and in complex oligonucleotide probe mixtures for detecting or localizing nucleic acid target genes within a cell or tissue sample. The development of such generic sequences and labeling strategy for probe collections has wide application in the medical, genetic, and molecular biological arts.

20 This interference due to labeling chemistry and hybridization stringency and kinetics is solved herein by designing the oligo to have at least two distinct functional domains, one domain or sequence to be gene specific and involved in the base pair formation, and the second domain to be an artificial, non-specific sequence (in reference to the sample's genome) comprised of spacing nucleotides and the labeled nucleotide. These elements are positioned so that these label-nucleotides are more accessible as haptens for binding proteins (immunoglobulin or avidin(s)) and thus do not interfere with Watson-Crick base pairing in the gene-specific domain.

#### SUMMARY OF THE INVENTION

30 The present invention provides a novel strategy to incorporate label into oligonucleotide probes and labeled oligonucleotide probe collections for detecting or

WO 02/22874

PCT/US01/28014

4

localizing nucleic acid target genes within a cell or tissue sample. In particular, the invention relates to non-gene-specific sequences using sequence formulas for making repetitive polymers of such sequences which can be incorporated into collections of oligonucleotide probes for use in *in situ* hybridization analyses. In addition, using labeled synthetic oligonucleotide polymers, based on sequence formulas, when conjugated to binding proteins, i.e. immunoglobulins, is a very effective and controlled process for labeling such proteins used in immunohistochemical analysis. The present invention provides collections or "cocktails" of oligonucleotide probes for detecting or localizing specific nucleic acid target genes within a cell or tissue sample. The cocktails are useful for detecting the following: the Kappa gene (SEQ ID NOS: 1-16 inclusive); the Lambda gene (SEQ ID NOS: 501-509, 511-513, and 515); the CMV (cytomegalovirus) gene (SEQ ID NOS: 221-241 inclusive); EBER (Epstein-Barr early RNA) gene (SEQ ID NOS: 51-54 inclusive); Alu (SEQ ID NOS: 55-56); PolyA (SEQ ID NO: 57); and the detection tail (SEQ ID NO:330).

The invention is directed to an oligonucleotide label-domain comprising the sequence (CTATTT)<sub>n</sub> and its complement (AAAATAG)<sub>n</sub>, wherein "n" is at least 1.

The invention is also directed to an oligonucleotide probe having at least two distinct functional domains, a first domain comprising the label-domain of claim 2, and a second domain comprising a gene-specific target sequence.

The invention is also directed to a probeset for detecting Kappa immunoglobulin light chain mRNA or corresponding heteronuclear RNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 401 through 416, inclusive.

The invention is also directed to a probeset for detecting Lambda immunoglobulin light chain mRNA or corresponding heteronuclear RNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 501 through 509, 511-513, and 515.

The invention is also directed to A probeset for detecting cytomegalovirus (CMV) immediate early RNA and/or corresponding mRNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 221 through 241

WO 02/22874

PCT/US01/28014

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The invention is also directed to a probeset for detecting Epstein Barr virus (EBV) early RNA, RNA 1 and RNA 2, (EBER) wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 51 through 54.

The invention is also directed to a probeset for detecting Human Alu repetitive satellite genomic DNA sequences wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 301 and 302.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

#### 10 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a generic probe structure of the two-domain probe design. This is the oligonucleotide design used for the probes in the gene specific cocktails described in the following examples. Each probe is composed of two domains: a 5' labeling domain and a 3' target gene target gene-specific domain. The labeling domain consists of this specific  
15 sequence (CTATTT)<sub>n</sub>, wherein each cytosine may be labeled with a fluorophore or a cytosine-hapten conjugate, the hapten being fluorescein in this embodiment. This illustration specifically shows nucleic acid sequences for the 301 (SEQ ID NO: 55) and 302 (SEQ ID NO: 56) probes, each of which possesses target gene gene-specific domains corresponding to human repetitive Alu sequences and labeling domains having a fluorescein hapten.

20 Figure 2 illustrates the results obtained for *in situ* hybridization (ISH) analysis of human skin tissue using a probe comprising the labeling domain (330 probe; SEQ ID NO: 58). The absence of a detectable signal indicates that the sequence formula, (CTATTT)<sub>n</sub>, of the labeling domain common to the oligonucleotides used in these ISH examples is non-specific, and non-reactive in its ability to form Watson-Crick base pairing with human  
25 nucleic acid sequences because it does not hybridize.

Figure 3 illustrates the results obtained for ISH analysis of human skin tissue using a probe comprising the labeling domain and a poly d(T) target gene-specific domain (320 probe; SEQ ID NO: 57). The presence of a detectable signal localized to the cytoplasm

WO 02/22874

PCT/US01/28014

6

indicates that this probe is capable of specifically hybridizing to polyadenylated region of messenger RNA.

Figures 4A-4B illustrate the results obtained for ISH analysis of human skin tissue using the 320 probe, wherein the tissue sample was not treated with ribonuclease A prior to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The decrease in detectable signal in (B) indicates that this probe specifically hybridizes to polyadenylated region common to messenger RNA.

Figures 5A-5B illustrate the results obtained for ISH analysis of human spleen tissue using the 320 probe, wherein the hybridization and stringency wash were performed at room temperature (A), or at 37°C (B). This result illustrates that the intensity of color is related to the stringency of hybridization conditions, with the more intense color indicating less stringent conditions.

Figure 6 illustrates the results obtained for ISH analysis of the human Raji cell line using the 320 probe. This shows that this probe design also is functional with embedded cell lines as well as embedded tissue.

Figure 7 illustrates the results obtained for ISH analysis of the human Raji cell line using a probe collection consisting of the 301 and 302 probes.

Figure 8 illustrates the results obtained for ISH analysis of the human HT cell line using a probe collection consisting of the 301 and 302 probes.

Figure 9 illustrates the results obtained for ISH analysis of a rat cell line using a probe collection consisting of the 301 and 302 probes. The absence of a detectable signal indicates that this probe collection is specific for human nucleic acid sequences.

Figure 10 illustrates the results obtained for ISH analysis of an Epstein-Barr virus (EBV)-negative human HT cell line using a probe possessing a target gene-specific domain corresponding to EBV EBER nuclear RNA [SEQ ID NO: 51 through SEQ ID NO: 54].

Figure 11 illustrates the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54].

Figure 12 illustrates the results obtained for ISH analysis of human tonsil tissue using a probe collection consisting of probes possessing target gene-specific domains

WO 02/22874

PCT/US01/28014

7

corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54].

5        Figures 13A-13B illustrate the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54], wherein the tissue sample was not treated with ribonuclease A prior to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The decrease in detectable signal in (B) indicates that this probe specifically hybridizes to human EBER 1 and EBER 2 nuclear RNA.

10        Figure 14 illustrates the results obtained for ISH analysis of kappa light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NO:15].

15        Figure 15 illustrates the results obtained for ISH analysis of lymphoma tissues using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin kappa light chain mRNA [SEQ ID NOS: 2 - 4, SEQ ID NOS:7-12, SEQ ID NOS: 14, 15]. The lymphoma tissue in (A) over expresses the kappa light chain and the tissue in (B) over expresses the lambda light chain. The absence of a detectable signal in (B) indicates that the kappa light chain probe collection is specific to kappa light chain mRNA.

20        Figure 16 illustrates the results obtained for ISH analysis of lambda light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain variable region mRNA [SEQ ID NOS:19 through 29].

25        Figure 17 illustrates the results obtained for ISH analysis of a lambda light chain-positive human RPMI 8226 cell line using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NOS:19 through 29].

30        Figures 18A-18B illustrate the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NOS:19 through 29]. The tissue in (A) over expresses the lambda light chain and the tissue in (B)

WO 02/22874

PCT/US01/28014

8

over expresses the kappa light chain. The absence of a detectable signal in (B) indicates that the lambda light chain probe collection is specific to human lambda light chain mRNA.

Figure 19 illustrates the results obtained for ISH analysis of cytomegalovirus (CMV)-positive human lung tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA [SEQ ID NOS:30-32,  
5 SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50]. [CMV infected cell]

Figure 20 illustrates the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has not been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-specific domains  
10 corresponding to CMV immediate early mRNA [SEQ ID NOS:30-32, SEQ ID NOS: 34-35,  
SEQ ID NO: 38, SEQ ID NO: 50].

Figures 21A-21B illustrate the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-specific domains  
15 corresponding to CMV immediate early RNA [SEQ ID NOS:30-32, SEQ ID NOS: 34-35,  
SEQ ID NO: 38, SEQ ID NO: 50]. The tissue in (A) is shown at a magnification of 40X and the tissue in (B) is shown at a magnification of 20X.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

20 The present invention provides oligonucleotide probes and oligonucleotide probe collections for detecting or localizing nucleic acid target genes within a cell or tissue sample. In particular, the invention relates to collections of oligonucleotide probes for use in *in situ* hybridization analyses.

More specifically, this invention relates to the use of specific sequence formulas for  
25 nucleotide polymers or label-domains to attach a detectable moiety (a label) to oligonucleotide probes or proteins. The specific utility of these sequences or derivatives thereof, is the inert or non-reactive characteristic that does not hybridize to human DNA or RNA at a detectable level under standard stringency of hybridization conditions. These label-domains or polymers were demonstrated to be useful generic sequences for  
30 incorporation into oligonucleotide probes for detecting gene-specific sequences within cells or tissue samples in *in situ* hybridization analyses. Additionally, this inert set of sequences

WO 02/22874

PCT/US01/28014

9

are useful for attaching a label to immunoglobulins or other proteins for detecting haptens and antigens in immunohistochemical analyses.

As used herein, the terms "probe" or "oligonucleotide probe" refers to a nucleic acid molecule used to detect a complementary nucleic acid target gene.

5 As used herein, the term "hybridization" refers to the process whereby complementary nucleic acid sequences join to form a double-stranded nucleic acid molecule. By labeling the target nucleic acid molecule with, for example, a radioactive or fluorescent tag, interactions between probe and target genes can be detected.

10 The oligonucleotide probes and oligonucleotide probes of the collections of the present invention are synthesized using conventional methods. See e.g., *Methods in Molecular Biology, Vol 20: Protocols for Oligonucleotides and Analogs* 165-89 (S. Agrawal, ed., 1993); *Oligonucleotides and Analogues: A Practical Approach* 87-108 (F. Eckstein, ed., 1991).

15 In a preferred embodiment of the present invention, oligonucleotide probes possess two distinct domains: a 5' (or labeling) domain and a 3' (or gene-specific target) domain (See Figure 1A). In more preferred embodiments of the present invention, the oligonucleotide probe possesses a labeling domain which consists of the sequence (CTATTT)<sub>n</sub>. Other embodiments are also demonstrated herein, including a triple-domain  
20 embodiment having two terminal labeling domains, and a central gene-specific target domain. Specifically, SEQ ID NOS: 125-126 depict this labeling scheme. Yet a further preferred embodiment of a labeling domain is TC(TTTTATC)<sub>n</sub> or its complement. This sequence is predicted to be as unique as the (CTATTT)<sub>n</sub> label-domain. The oligonucleotide probes of the present invention are labeled so that hybridization between said  
25 probes and target nucleic acids in a particular cell or tissue can be detected. Labels that are acceptable for use in *in situ* hybridization (ISH) analysis are known to those with skill in the art. Such labels permit interactions between probe and target genes to be detected using, for example, radioactive-based direct detection methods, fluorescence-based direct detection  
30 methods, digoxigenin-labeled or biotin-labeled probes coupled with fluorescence-based detection methods, or digoxigenin-labeled or biotin-labeled probes coupled with antibody-enzyme-based detection methods. In preferred embodiments of the present invention, oligonucleotide probes are labeled with fluorescein. In more preferred embodiments of the

WO 02/22874

PCT/US01/28014

10

present invention, the oligonucleotide probe possesses a labeling domain which consists of the sequence (CTATTTT)<sub>n</sub>, wherein the cytosine nucleotides may be labeled with a fluorophore for direct detection, or a hapten for indirect detection. In either, the fluorescein-cytosine nucleotide conjugate and the fluorescein molecule is linked at the N4 position of cytosine through an OBEB linkage (See Mishra *et al.*, U.S. Patent No. 5,684,142, which is incorporated herein by reference). In a preferred embodiment, the density of fluorophore attached to the label-domain is at least 7 mole percent, preferably at least 10 mole percent, and most preferably at least 16 mole percent, when measured against the label-domain solely. For example, if probe 401 is considered (a 2-domain probe) it comprises a label-domain of 30 bases including a 3' terminal CT wherein the C is also labeled, the mole percent is 5/30 = 16.7 mole percent label. In the overall probe, the mole percent is 8.3.

In some embodiments of the present invention, several target genes are simultaneously analyzed by exposing a cell or tissue sample to a plurality of nucleic acid probes that have been labeled with a plurality of different nucleic acid tags. For example, a plurality of nucleic acid probes can be labeled with a plurality of fluorescent compounds having different emission wavelengths, thereby permitting simultaneous multicolored analysis to be performed in a single step on a single target cell or tissue sample.

The oligonucleotide probes and oligonucleotide probe collections of the present invention may be used in ISH analysis to detect or localize nucleic acid target genes within a cell or tissue sample. ISH may be performed as described, for example, in *In Situ Hybridization: Medical Applications* (G. R. Coulton and J. de Bellerche, eds., Kluwer Academic Publishers, 1992); *In Situ Hybridization: In Neurobiology; Advances in Methodology* (J. H. Eberwine, K. L. Valentino, and J. D. Barchas, eds., Oxford University Press, 1994); or *In Situ Hybridization: A Practical Approach* (D. G. Wilkinson, ed., Oxford University Press, 1992).

The preferred embodiment of the probes and probe collections of the present invention are best understood by referring to Figures 1-21 and Examples 1-2. The Examples, which follow, are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

WO 02/22874

PCT/US01/28014

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**EXAMPLE 1****Probe Collection Preparation**

Probe collections consisting of a plurality of oligonucleotide probes of 55 to 60 bases in length were designed as follows. In this Example, each oligonucleotide probe possessed two distinct domains: a 5' (or labeling) domain and a 3' (or target gene-specific) domain (See Figure 1).

In this embodiment, the labeling domain consists of the sequence (CTATTT)<sub>n</sub>, wherein the cytosine nucleotide represents a fluorescein-cytosine nucleotide conjugate and the fluorescein molecule is linked at the N4 position of cytosine through an OBBA linkage.

The target gene-specific domain consists of a 25-30 base sequence that is complementary to a specific nucleic acid target gene. Oligonucleotide probes were designed to possess target gene-specific domains corresponding to the human immunoglobulin kappa light chain variable region (See Table 1; oligonucleotide probes 401-416), the human immunoglobulin lambda light chain variable region (oligonucleotide probes 501-515), human cytomegalovirus (CMV) sequences (oligonucleotide probes 221-241), human Epstein-Barr virus (EBV) EBER (Epstein-Barr early RNA) sequences (oligonucleotide probes 100A2, 100C2, 100A1, and 100B1), human repetitive Alu sequences (oligonucleotide probes 301 and 302), and poly d(T) (oligonucleotide probe 320).

**EXAMPLE 2****Label-domain design: Alu repetitive sequence probe**

Four probes all against the Alu human repetitive sequence were used to evaluate label-domain design. The probes numbered 301 (SEQ ID NO: 55), 301A (SEQ ID NO: 116), 301A2/2 (SEQ ID NO: 121), and 301A3/2 (SEQ ID NO: 122) are shown in Table 1.

The four probes were evaluated at the concentrations of 100, 75, 50, and 25 ng/ml per mL of probe in the reaction, respectively. This hybridization analysis was done manually, using standard protocols. The target, paraffin-embedded cell line MBA MD 468 (Oncor INFORM™ Her-2/neu Control Slides, Cat. No. S8100, Level 1, available from Ventana Medical Systems, Inc., Tucson, AZ) was the target sample and was processed by removing paraffin by standard xylene methods. The tissue was subjected to Ventana's Protease 1 for 12 minutes at 50 degrees C as a 1:2 dilution with Ventana's APK buffer. The hybridization reaction was accomplished with the addition of probe diluent as 100 ul probe (25%

WO 02/22874

PCT/US01/28014

12

formamide, 5% dextran sulfate, 2X SSC, 1% Triton) to a residual 100 ul volume of 2XSSC/Triton X-100. The slide was heated to 85 degrees C for 5 minutes and then incubated for 1 hr at 37 degrees C. Standard SSC washes followed for removing excess probe. The hybrids were detected with an antibody against FITC. The mouse antibody was detected colometrically using Ventana Enhanced Alkaline Phosphatase Blue Detection (cat# 760-061). Unless otherwise indicated, all reagents were obtained from Ventana Medical Systems, Inc., Tucson, AZ. The results were observed by colormetric detection using brightfield microscopy.

The results of these experiments were that signal intensity was a function of the total number of fluorescein hapten conjugated to the probe and signal was of the specific label-domain design. The greater the number of fluoresceins per probe molecule, the greater the signal observed. Comparison of design and placement of haptens on the probe showed that this was not a factor in signal intensity. The two probes that contained five fluoresceins, (301A3/2 (SEQ ID NO: 122) and 301 (SEQ ID NO:55) both yielded equivalent signal. These two probes yielded greater signal than seen for 301A2/2, a probe with a split label-domain design with four fluoresceins. The probe 301A2/2 yielded a signal greater than probe 301A a probe with a single label-domain design at the 5' end and with three fluoresceins.

### EXAMPLE 3

#### 20 Label Domain Design: EBER probes

This experiment compared two label-domain designs and sequences to determine whether greater spacing between the fluorescein haptens improves the production of signal during probe detection steps during *in situ* hybridization analysis.

The tissue used was an EBV-infected human spleen tissue fixed in neutral buffered formalin paraffin embedded section of 4-micron thickness placed on silane plus glass microscope slides. The tissue sections were deparaffinized on a Ventana DISCOVERY™ machine, followed by a 6-min digestion with Ventana's Protease 1, at a temperature of 37 C. The probe was dissolved in hybridization buffer diluent at a concentration of 50 ng/mL as a 100 ul applied to an equal volume of 2 X SSC/ Triton X-100 residual volume left on the slide after prepared by the Ventana Medical Systems, Inc. automated ISH staining system, Discovery. The probe diluent-mixed with the residual volume on slide for 6 min at 37 C,

WO 02/22874

PCT/US01/28014

/3

then the solution was heated to 85C and held there for a total of 10 min. The slide was then taken to a 37C temperature and held at that temperature for 1 hour. All of these aqueous reactions on the slide were all done under a film of LIQUID COVERSLIP™, to prevent evaporative loss of water during processing. Each slide after hybridization was washed 3 times with 2X SSC/Triton solution, with a 6 min incubation between each wash, the slide volume being approximately 300 ul (+/- 10% vol). The hybrids were detected with an antibody against FITC. The mouse antibody was detected colometrically using Ventana Enhanced Alkaline Phosphatase Blue Detection (cat# 760-061).

The two oligonucleotide probes used for this study probe 100A1 (SEQ ID NO: 53) and 1002A32 (SEQ ID NO: 120). The two differences between these probes were the label-domain sequence and structure. The probe 100A1 label domain was 5' to gene target domain, contained 5 fluoresceins attached to cytosine residues via the OBEA linker, with the sequence formula of (CTATTTT)<sub>4</sub>CT (SEQ ID NO: 58). The label domain of the oligo probe 1002A32, was similar, (SEQ ID NO:125). Besides the different sequence the primary difference was that the fluoresceine labeled cytosines were spaced 10 bases apart compared to the oligo 100A1 the cytosine spacing was closer at 7 bases apart. The result of this comparison as deduced by H score analysis were that these oligonucleotide were equivalent as to the amount of signal generated on the slide. The data was that for 100A2, for the 368 cells analysed in a total of 3 fields the H score was 106, and for probe 1002A32 for the 345 cell analysed in three field the H score was 109. The H score is a spectrographic analysis done with microscope that factors into the score background to signal ratio on the tissue section to yield a relative comparison of total target specific signal on the slide. (See reference Giroud, F. Perrin C, and Simony Lafontaine, J.; Quantitative Immunocytochemistry and Immunohistochemistry. Third Conference of the European Society for Analytical Cellular Pathology, 1994; and AutoCyte Quic Immuno User's Manual, 1998, document number PA-029, Co AutoCyte Inc. Burlington NC 2721). The histograms and the score sheet indicated that each oligo were equally efficient in yielding a colometric signal. This indicates that the position of the label domain can be either 3 prime or 5 prime to the gene target sequence or the gene target sequence can be positioned between two label domains.

#### EXAMPLE 4

##### In Situ Hybridization

WO 02/22874

PCT/US01/28014

14

The probe collections prepared in Example 1 were first diluted in a solution consisting of 20% dextran sulfate (wt/vol), 50% formamide (vol/vol), 2X SSC, 10 mM Tris-HCl, 5 mM EDTA, and 0.05% Brij-35, at a final pH of 7.3. Probe collections were then mixed with an equal volume of a solution consisting of 2X SSC and 0.05% Triton X-100.

5 Samples for ISH analysis were prepared by cutting formalin-fixed and paraffin-embedded cells or tissue samples into 4  $\mu$ m sections and placing the sections onto a glass slide. Subsequent processing and ISH of samples was carried out in an automated device, such as the DISCOVERY™ Automated ISH/IHC Stainer (Ventana Medical Systems, Inc., Tucson, AZ) described in co-owned and co-pending U.S. Patent App. Serial Nos. 60/076,198 and 09/259,240, both incorporated herein by reference. To remove paraffin from the samples, the slides were immersed in an aqueous solution, heated for approximately 20 minutes, and then rinsed. The automated deparaffinization procedure is more fully described in U.S. Serial No. 60/099,018, 09/259,240 both incorporated herein by reference. The samples were then treated with protease and the slides were heated to 85°C (for hybridization to RNA target genes) or 90-95°C (for hybridization to DNA target genes) for 4 to 10 minutes.

15 Hybridization reactions were typically performed in a hybridization buffer consisting of 10% dextran sulfate (wt/vol), 25% formamide (vol/vol), 2X SSC, 5 mM Tris, 2.5 mM EDTA, 0.025% Brij-35, 0.25% Triton X-100, and between 25 to 125 ng/mL of each individual probe molecule. ISH reactions were performed at between 37°C to 54°C. For ISH using the probe collections described in Example 1, hybridization reactions were optimally carried out for 1 hr at 47°C (except for the poly d(T) probe, wherein the hybridization reaction was optimally carried out at 37°C for 1 hr).

20 The hybridization of fluorescein-labeled probe molecules to a particular target gene in the sample was detected by using a sequential series of binding proteins, i.e., secondary antibody detection. However, it is equally possible to use direct detection when visualizing the bound probes. In secondary detection, first, an anti-fluorescein mouse monoclonal antibody directed against the fluorescein-labeled probe molecule was added to the sample. Next, a biotin-labeled polyclonal goat antibody directed against the mouse antibody was added to the sample. Finally, hybridization reactions were colorimetrically detected using a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate. This technique, termed "secondary antibody detection," is routine for one of skill in the art.

WO 02/22874

PCT/US01/28014

15

Primary and secondary antibodies are available from numerous suppliers, including Ventana Medical Systems, Tucson, AZ, which are optimized for use on the Ventana autostaining systems (ES<sup>®</sup>, NexES<sup>®</sup>, DISCOVERY<sup>™</sup>, and BENCHMARK<sup>™</sup>).

5 Figures 2-21 illustrate the results obtained for *in situ* hybridization analysis of various cell lines or tissue samples using the probes disclosed and claimed herein having the structural motif illustrated in Figure 1 or probe collections consisting of such probes.

Figure 1 illustrates a generic probe structure of the two-domain probe design. This is the oligonucleotide design used for the probes in the gene specific cocktails described in the following examples. Each probe is composed of two domains: a 5' labeling domain and a 3' target gene target gene-specific domain. The labeling domain consists of this specific sequence (CTATTTT)<sub>n</sub>, wherein the cytosine nucleotide is a cytosine-hapten conjugate, the hapten being fluorescein in this embodiment. This illustration specifically shows nucleic acid sequences for the 301 (SEQ ID NO: 55) and 302 (SEQ ID NO: 56) probes, each of which possesses target gene gene-specific domains corresponding to human repetitive Alu sequences and labeling domains having a fluorescein hapten.

Figure 2 illustrates the results obtained for *in situ* hybridization (ISH) analysis of human skin tissue using a probe comprising the labeling domain (330 probe; SEQ ID NO: 58). The absence of a detectable signal indicates that the sequence formula, (CTATTTT)<sub>n</sub>, of the labeling domain common to the oligonucleotides used in these ISH examples is non-specific, and non-reactive in its ability to form Watson-Crick base pairing with human nucleic acid sequences because it does not hybridize.

Figure 3 illustrates the results obtained for ISH analysis of human skin tissue using a probe comprising the labeling domain and a poly d(T) target gene-specific domain (320 probe; SEQ ID NO: 57). The presence of a detectable signal localized to the cytoplasm indicates that this probe is capable of specifically hybridizing to polyadenylated region of messenger RNA.

Figures 4A-4B illustrate the results obtained for ISH analysis of human skin tissue using the 320 probe, wherein the tissue sample was not treated with ribonuclease A prior to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The decrease in detectable signal in (B) indicates that this probe specifically hybridizes to polyadenylated region common to messenger RNA.

WO 02/22874

PCT/US01/28014

16

Figures 5A-5B illustrate the results obtained for ISH analysis of human spleen tissue using the 320 probe, wherein the hybridization and stringency wash were performed at room temperature (A), or at 37°C (B). This result illustrates that the intensity of color is related to the stringency of hybridization conditions, with the more intense color indicating less stringent conditions.

Figure 6 illustrates the results obtained for ISH analysis of the human Raji cell line using the 320 probe. This shows that this probe design also is functional with embedded cell lines as well as embedded tissue.

Figure 7 illustrates the results obtained for ISH analysis of the human Raji cell line using a probe collection consisting of the 301 and 302 probes.

Figure 8 illustrates the results obtained for ISH analysis of the human HT cell line using a probe collection consisting of the 301 and 302 probes.

Figure 9 illustrates the results obtained for ISH analysis of a rat cell line using a probe collection consisting of the 301 and 302 probes. The absence of a detectable signal indicates that this probe collection is specific for human nucleic acid sequences.

Figure 10 illustrates the results obtained for ISH analysis of an Epstein-Barr virus (EBV)-negative human HT cell line using a probe possessing a target gene-specific domain corresponding to EBV EBER nuclear RNA [SEQ ID NO: 51 through SEQ ID NO: 54].

Figure 11 illustrates the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54].

Figure 12 illustrates the results obtained for ISH analysis of human tonsil tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54].

Figures 13A-13B illustrate the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to EBV EBER 1 and 2 nuclear RNA [SEQ ID NO:51 through SEQ ID NO:54], wherein the tissue sample was not treated with ribonuclease A prior to *in situ* hybridization (A), or was treated with ribonuclease A prior to *in situ* hybridization (B). The

WO 02/22874

PCT/US01/28014

17

decrease in detectable signal in (B) indicates that this probe specifically hybridizes to human EBER 1 and EBER 2 nuclear RNA.

Figure 14 illustrates the results obtained for ISH analysis of kappa light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NO:15].

Figure 15 illustrates the results obtained for ISH analysis of lymphoma tissues using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin kappa light chain mRNA [SEQ ID NOS: 2 - 4, SEQ ID NOS:7-12, SEQ ID NOS: 14, 15]. The lymphoma tissue in (A) over expresses the kappa light chain and the tissue in (B) over expresses the lambda light chain. The absence of a detectable signal in (B) indicates that the kappa light chain probe collection is specific to kappa light chain mRNA.

Figure 16 illustrates the results obtained for ISH analysis of lambda light chain-positive human tonsil tissue using a probe possessing a target gene-specific domain corresponding to human immunoglobulin lambda light chain variable region mRNA [SEQ ID NOS:19 through 29].

Figure 17 illustrates the results obtained for ISH analysis of a lambda light chain-positive human RPMI 8226 cell line using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NOS:19 through 29].

Figures 18A-18B illustrate the results obtained for ISH analysis of human spleen tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to human immunoglobulin lambda light chain mRNA [SEQ ID NOS:19 through 29]. The tissue in (A) over expresses the lambda light chain and the tissue in (B) over expresses the kappa light chain. The absence of a detectable signal in (B) indicates that the lambda light chain probe collection is specific to human lambda light chain mRNA.

Figure 19 illustrates the results obtained for ISH analysis of cytomeglovirus (CMV)-positive human lung tissue using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA [SEQ ID NOS:30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50]. Arrow indicates CMV infected cell.

WO 02/22874

PCT/US01/28014

18

Figure 20 illustrates the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has not been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA [SEQ ID NOS:30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50].

Figures 21A-21B illustrate the results obtained for ISH analysis of a rat 9G cell line in which the expression of CMV immediate early RNA has been induced by cyclohexamide using a probe collection consisting of probes possessing target gene-specific domains corresponding to CMV immediate early RNA [SEQ ID NOS:30-32, SEQ ID NOS: 34-35, SEQ ID NO: 38, SEQ ID NO: 50] expression of the CMV immediate early RNA with cyclohexamide. The tissue in (A) is shown at a magnification of 40X and the tissue in (B) is shown at a magnification of 20X.

Table I

Probe ID	Sequence	SEQ ID
401	5'-CTATTTCTATTTCTATTTCTATTTCT CCAGAGTAGCAGGAGCCCCAGGAGCTGAGC-3'	1
402	5'-CTATTTCTATTTCTATTTCTATTTCT GGATGAGACTGGGTCACTGGATGTCAG-3'	2
403	5'-CTATTTCTATTTCTATTTCTATTTCT GCAGGAGATGGTACTCTGTCTCTACAGC-3'	3
404	5'-CTATTTCTATTTCTATTTCTATTTCT TCTGTCCAGATCCACTGCCACTGAACCTT-3'	4
405	5'-CTATTTCTATTTCTATTTCTATTTCT GCAGCCACAGTTGCTTCATCTGACCTTG-3'	5
406	5'-CTATTTCTATTTCTATTTCTATTTCT TTTCAACTGCTCTCATCGATGGCGGAGAT-3'	6
407	5'-CTATTTCTATTTCTATTTCTATTTCT AAGTTATTCAGCAGCCACACACAGAGGCA-3'	7
408	5'-CTATTTCTATTTCTATTTCTATTTCT GRCGTTATCPAGCTTCCACTGTACTTTGGC-3'	8
409	5'-CTATTTCTATTTCTATTTCTATTTCT TAGGTGCTGCTCTGTCTGCTGCTGCTG-3'	9
410	5'-CTATTTCTATTTCTATTTCTATTTCT GTAGTCTGCTTGTCTCAGCCTCAGGCTGCT-3'	10
411	5'-CTATTTCTATTTCTATTTCTATTTCT GATGGTGACTTCCAGGCTAGACTTTGT-3'	11
412	5'-CTATTTCTATTTCTATTTCTATTTCT CTCTCCCTGTTGAGCTCTTTGAGAGGG-3'	12
413	5'-CTATTTCTATTTCTATTTCTATTTCT TGGAACTGAGGAGCGTGGGGGACTTCT-3'	13
414	5'-CTATTTCTATTTCTATTTCTATTTCT GAAAAGGGTCAGAGGCCAAGGATGGGAG-3'	14
415	5'-CTATTTCTATTTCTATTTCTATTTCT AGATGAGCTGACGACCGCCAAAGGSGTAG-3'	15

19

WO 02/22874

PCT/US01/28014

416	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	GCATTAATTAAGCCAGGAGGAGGAGGAGG-3'	16
501	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	CCTGAGTGAGGAGGAGGAGGAGGAGGAGG-3'	17
502	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	AGACCCAGACACGGAGGAGGAGGAGGAG-3'	18
503	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	TGTTGGTCCAGTGCAGGAGATGGTATCG-3'	19
504	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	TAAATCATGATTTTGGGGGCTTTGGCTGGG-3'	20
505	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	TGTTGCCAGGCTTGGAGCCAGAGAGCGAT-3'	21
506	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	AATATCTGCGCTGCTCAGCCTGGAGCC-3'	22
507	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	GGTCCCTCCGCCGAAACACAGTGTACT-3'	23
508	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	TATGAGACACACCAGTGTGGGCTTGTGG-3'	24
509	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	CTGCTCAGGCGTCAAGCTCAGATAGCTGCT-3'	25
511	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	ATSCCTGACCTGGCAGCTGTAGCTTCTGTG-3'	26
512	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	ATTCTGTAGGGGCCACTGTCTTCTCCACGG-3'	27
513	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	CCTCCCTGGGATCCTGCAGTCTAGTCTC-3'	28
515	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	TGAGGGTTTATGAGTGCAGGGAGAGGSC-3'	29
221	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	GGAGGTCAAAACAGGCTGGATGGCG-3'	30
222	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	GGGCTGGATCGGTCCCGGTGTCTT-3'	31
223	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	AATCCGCTTCCCATGCACCGTTCG-3'	32
224	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	TAAATACTGGGGCACTGGGGAGCG-3'	33
225	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	ACCCGAGATTGCGGTGAGATCCCA-3'	34
226	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	GAGCAAGGAGCTCCGAGCCACAT-3'	35
227	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	ACACTGGTGGTGGGCACTGCTGC-3'	36
228	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	TTCGAATGCGTCAAGGCTGCAAGC-3'	37
229	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	AGCTGCTGCACTCTTCTGTCGCGC-3'	38

20

WO 02/22874

PCT/US01/28014

238	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	TCTCAGAGGATCGGCCCCCGAATG-3'	47
239	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	CCTCATCTGACTCCTCGGCGATGGC-3'	48
240	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	CGGGTACGGGGACTCTGGGGGTGA-3'	49
241	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	GGGTGGTTCCTTTGGCTCCGAGG-3'	50
100A2	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	GACCTCGGTCGGTAGCACCCGACT-3'	51
100C2	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	GGAGCCCTCTCTTCTCCCTCCCGG-3'	52
100A1	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	CCACGACACCCCTCCACCCACCCG-3'	53
100B1	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	GGCTACAGCCACACACCTCTCCCTCC-3'	54
301	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	CGAGGCGGCGGATCACCTGAGGC-3'	55
302	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	CGGAGGCGGAGGTTGCAGTGAGCC-3'	56
320	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	TTTTTTTTTTTTTTTTTTTTTTTTTT-3'	57
301A	5'-CTATTTTCATTTTCTTTT	CGAGGCGGCGGATCACCTGAGGC-3'	116
302C	5'-CTATTTTCATTTTCTTTT	CGGAGGCGGAGGTTGCAGTGAGCC-3'	117
302A4	5'-CTATTTTATACTTTATATTTTCAATTTTATCT	CGGAGGCGGAGGTTGCAGTGAGCC-3'	118
302A3/2	5'-CTATTTTATACTTTATATTTTCT	CGGAGGCGGAGGTTGCAGTGAGCC ACTATTTTATACTT-3	119
1002A32	5'-CTATTTTATACTTTATATTTTCT	GACCTCGGTCGGTAGCACCCGCAC TACTATTTTATACTT-3'	120
301A2/2	5'-CTATTTTCTTT	CGAGGCGGCGGATCACCTGAGGC TTCITTTTATCTT-3	121
301A3/2	5'-CTATTTTATACTTTATATTTTCT	CGGAGGCGGAGGATCACCTGAGGC ACTATTTTATACTT-3'	122

Table 2

5

Probe ID	Sequence	SBQ ID
	5'-CTATTTTCATTTTCTTTT	123
	5'-CTATTTTATACTTTTATATTTTCAATTTTATCT	124
330	5'-CTATTTTCATTTTCTATTTTCTATTTTCT	56
	5'-CTATTTTATACTTTTATATTTTCT . . . . . ACTATTTTATACTT-3	125
	5'-CTATTTTCTTT . . . . . TTTCTTTTATCTT-3	126

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

WO 02/22874

PCT/US01/28014

2/

## WE CLAIM:

1. An oligonucleotide label-domain comprising the sequence (CTATTT)<sub>n</sub> and its complement (AAAATAG)<sub>n</sub> wherein "n" is at least 1.  
5
2. The oligonucleotide label-domain of claim 1 detectably labeled with a reporter molecule, or a hapten molecule.
3. The oligonucleotide label-domain of claim 2 wherein the hapten is fluorecein linked to the N4 nitrogen of cytosine through an OBBA linker.  
10
4. The oligonucleotide label-domain of claim 1 wherein the reporter molecule is a fluorophore.
- 15 5. The oligonucleotide label-domain of claim 1 wherein the fluorophore is present at a density of greater than 7 mole percent.
6. The oligonucleotide label-domain of claim 1 wherein the label-domain has the sequence TC(TTTATC)<sub>n</sub> ( or its complementary formula).  
20
7. The oligonucleotide label-domain of claim 1 wherein the sequence is SEQ ID NO: 58.
8. The oligonucleotide label-domain of claim 2 wherein at least 7 mole percent of the cytosines are linked to a detectable moiety by an OBBA linker.

WO 02/22874

PCT/US01/28014

22

9. An oligonucleotide probe having at least two distinct functional domains, a first domain comprising the label-domain of claim 2, and a second domain comprising a gene-specific target sequence.
- 5
10. The oligonucleotide probe of claim 9 wherein the label-domain is located at the 5' end of the oligonucleotide probe, and the gene-specific target sequence being 3' to the label-domain.
- 10
11. The oligonucleotide probe of claim 9 wherein the label-domain is located at the 3' end of the oligonucleotide probe, and the gene-specific target sequence is 5' to the label-domain.
12. An oligonucleotide probe having three distinct functional domains, a first domain comprising the label-domain of claim 2, a second domain comprising a gene-specific target sequence, and a third domain comprising another label-domain, wherein said second domain is located between said first and third domains.
- 15
13. A probeset for detecting Kappa immunoglobulin light chain mRNA or corresponding heteronuclear RNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 401 through 416, inclusive.
- 20

WO 02/22874

PCT/US01/28014

23

14. A probeset for detecting Lambda immunoglobulin light chain mRNA or corresponding heteronuclear RNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 501 through 509, 511-513, and 515.
- 5 15. A probeset for detecting cytomegalovirus (CMV) immediate early RNA and /or corresponding mRNA wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 221 through 241
- 10 16. A probeset for detecting Epstein Barr virus (EBV) early RNA, RNA 1 and RNA 2, (EBER) wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 51 through 54.
- 15 17. A probeset for detecting Human Alu repetitive satellite genomic DNA sequences wherein the probes are selected from the group consisting essentially of SEQ ID NOS: 301 and 302.

# Oligonucleotide Probe Design

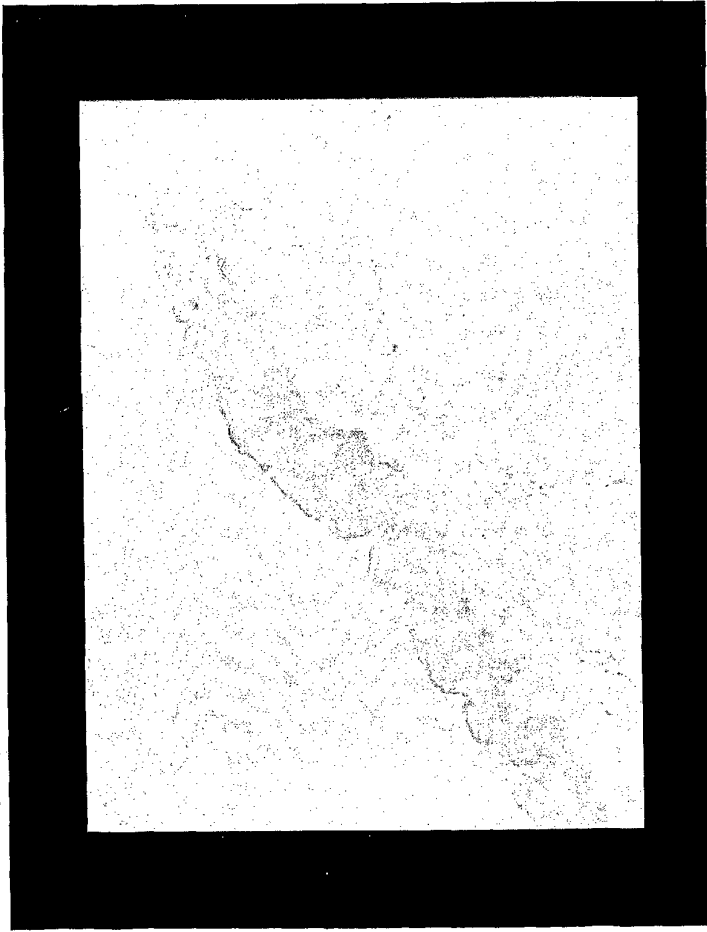
Common Signal Detection Region      Unique Target Detection Region

5'--3'

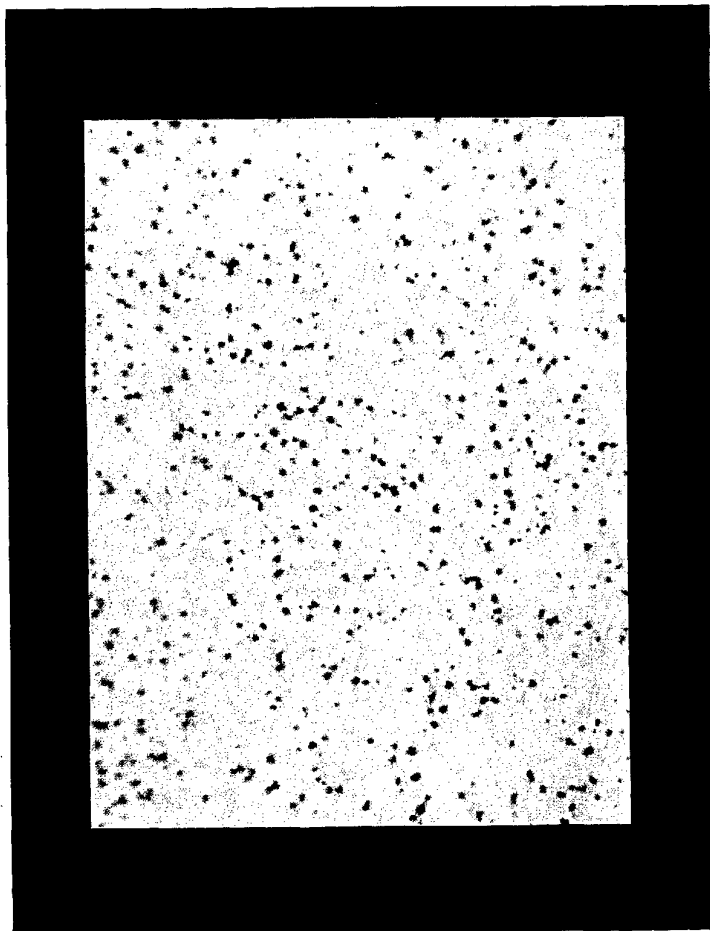
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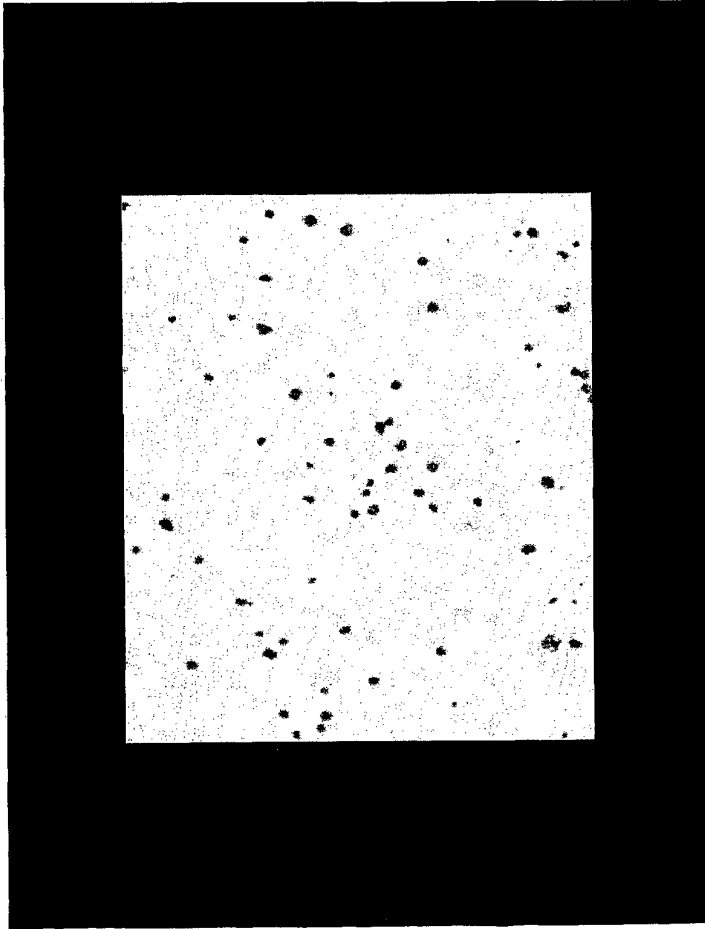
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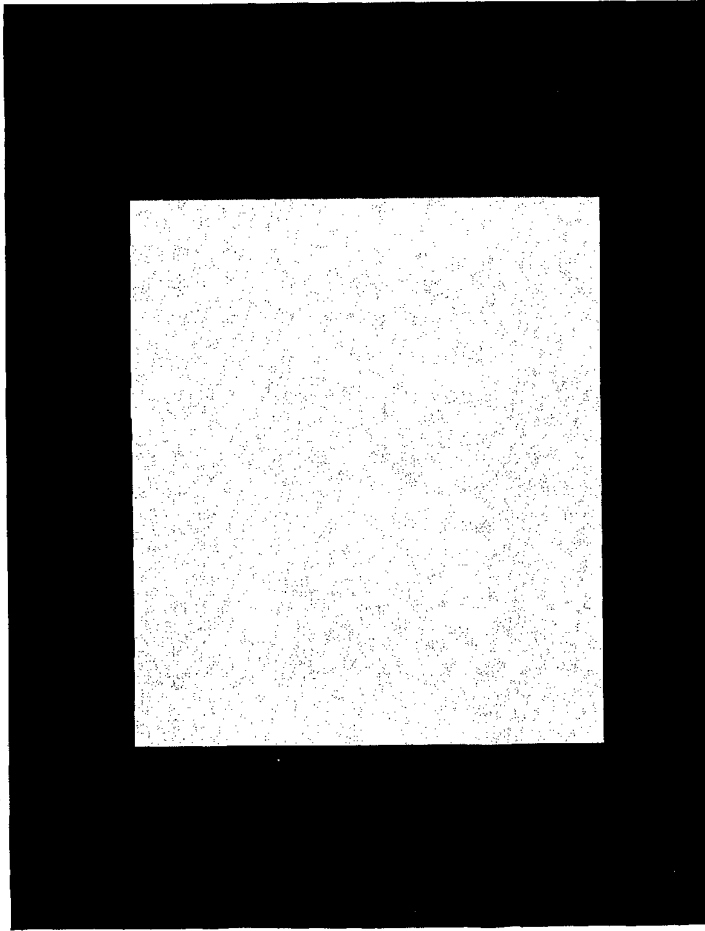
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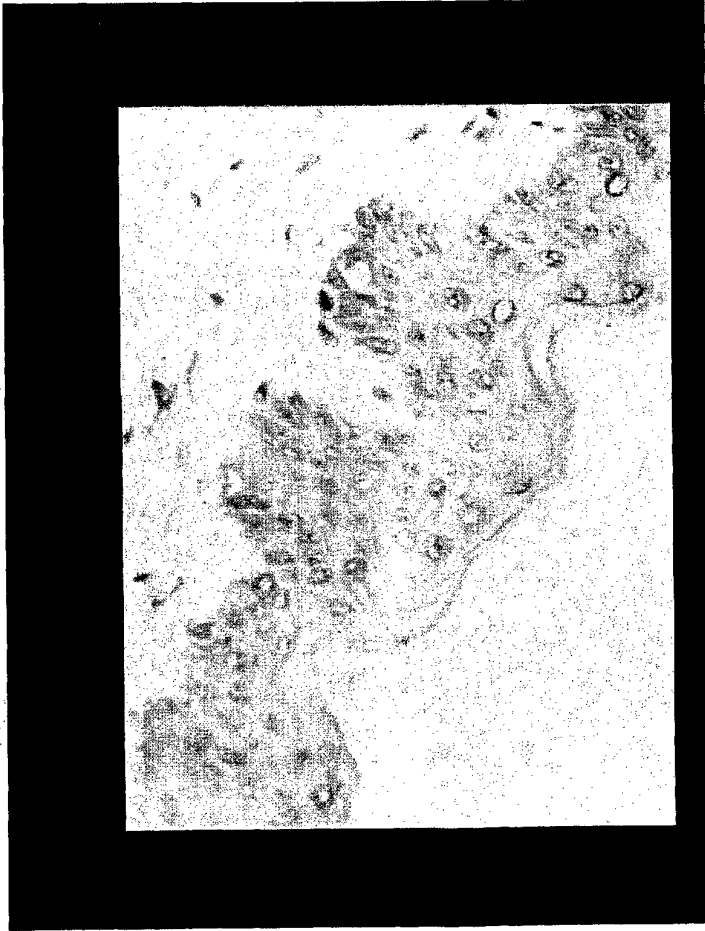


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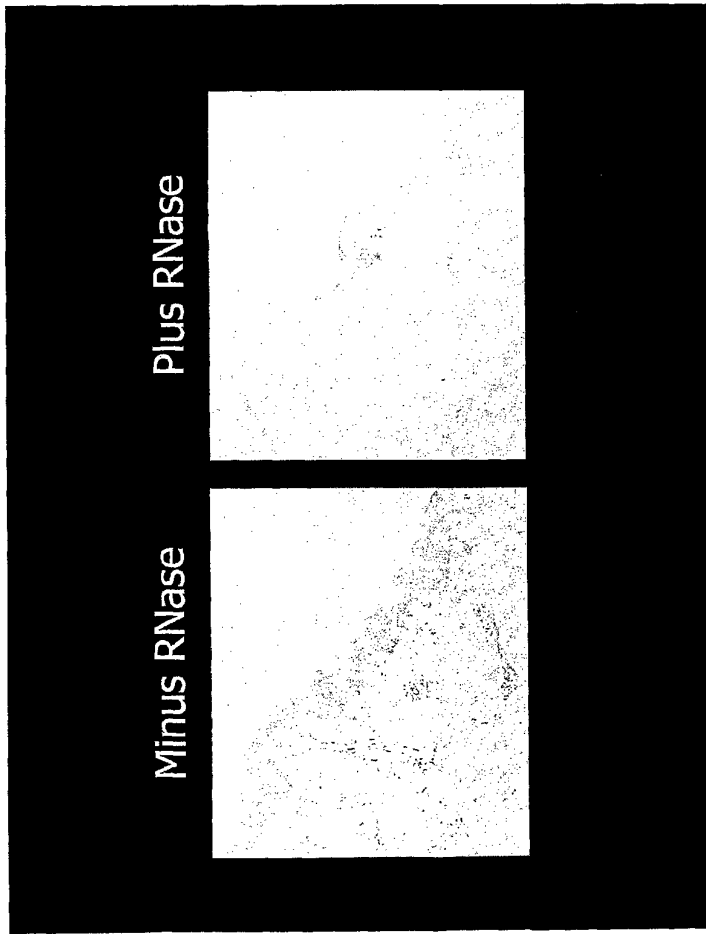


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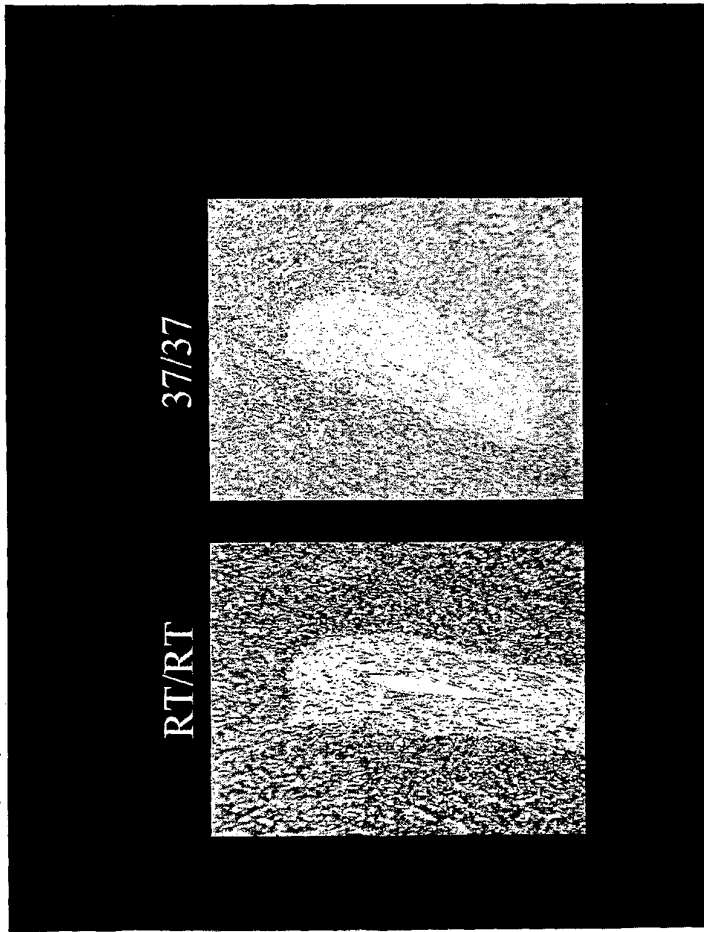


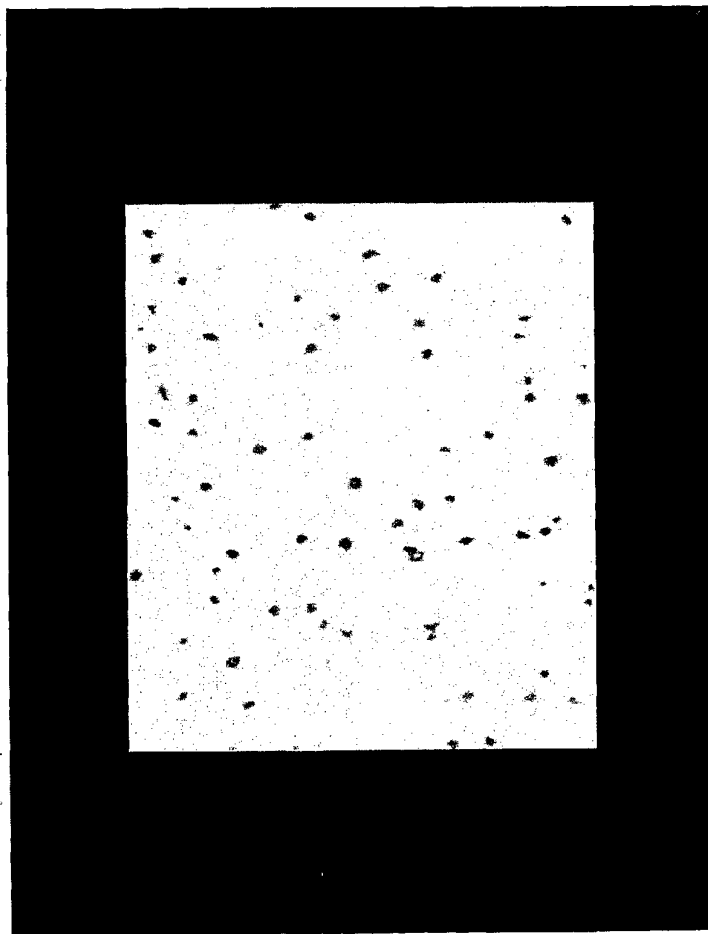


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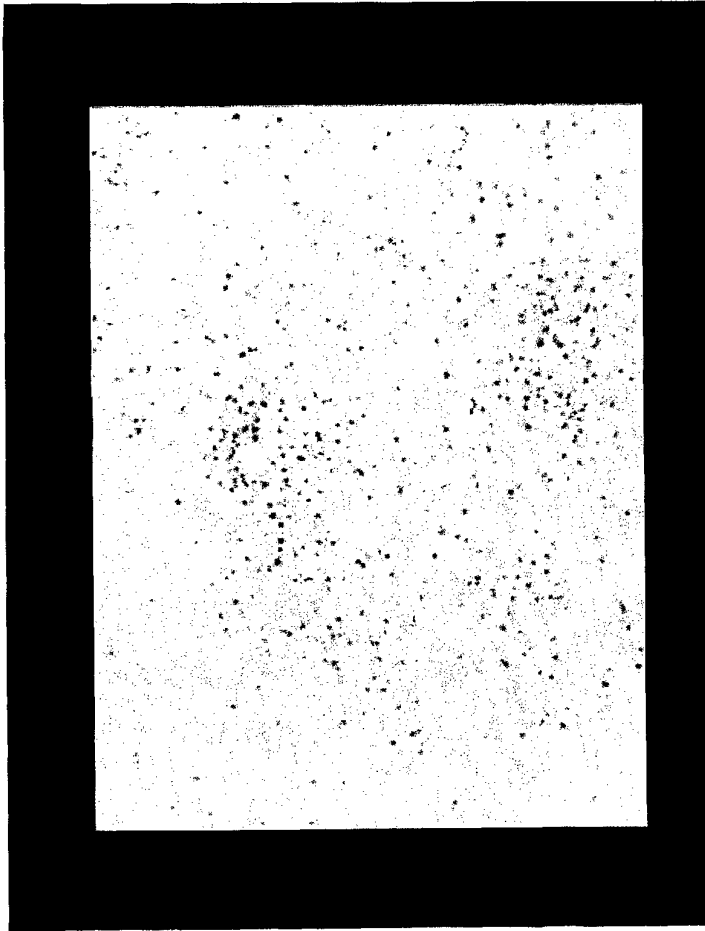


WO 02/22874

10/21

PCT/US01/28014

33

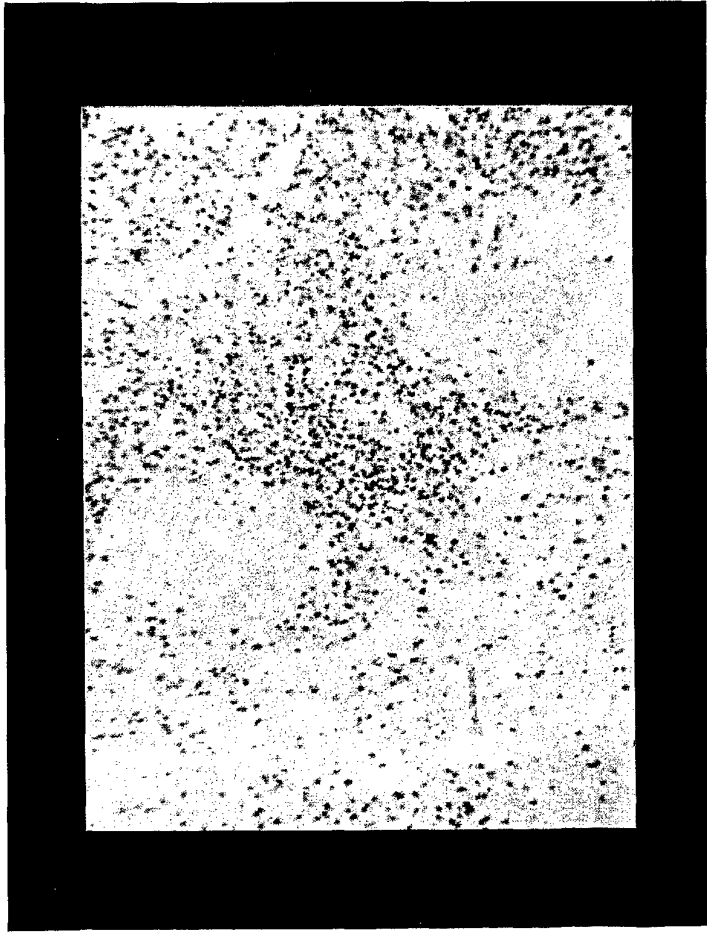


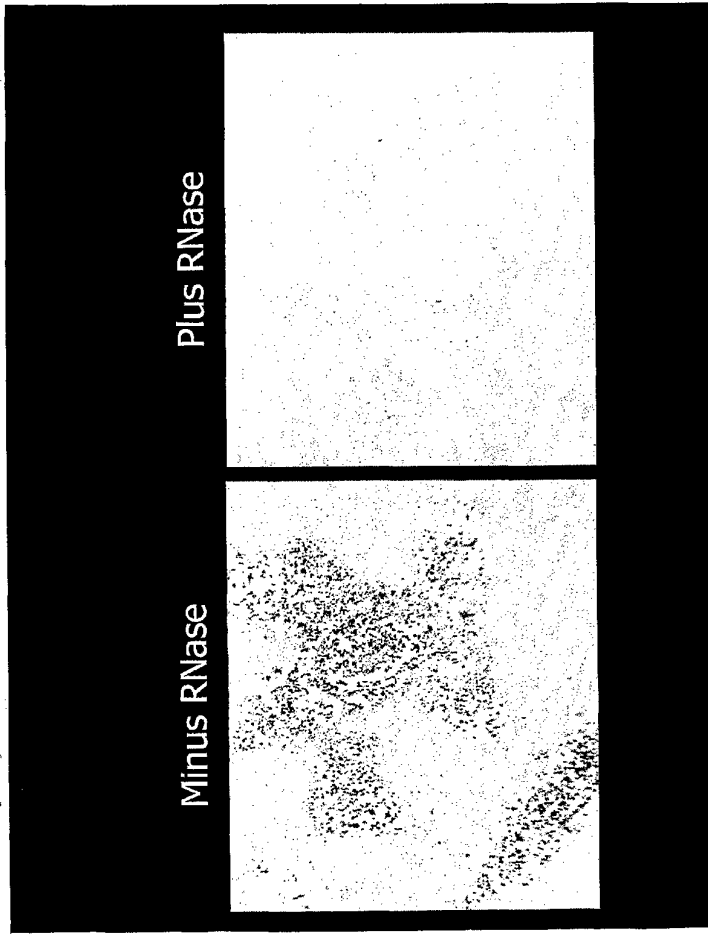
WO 02/22874

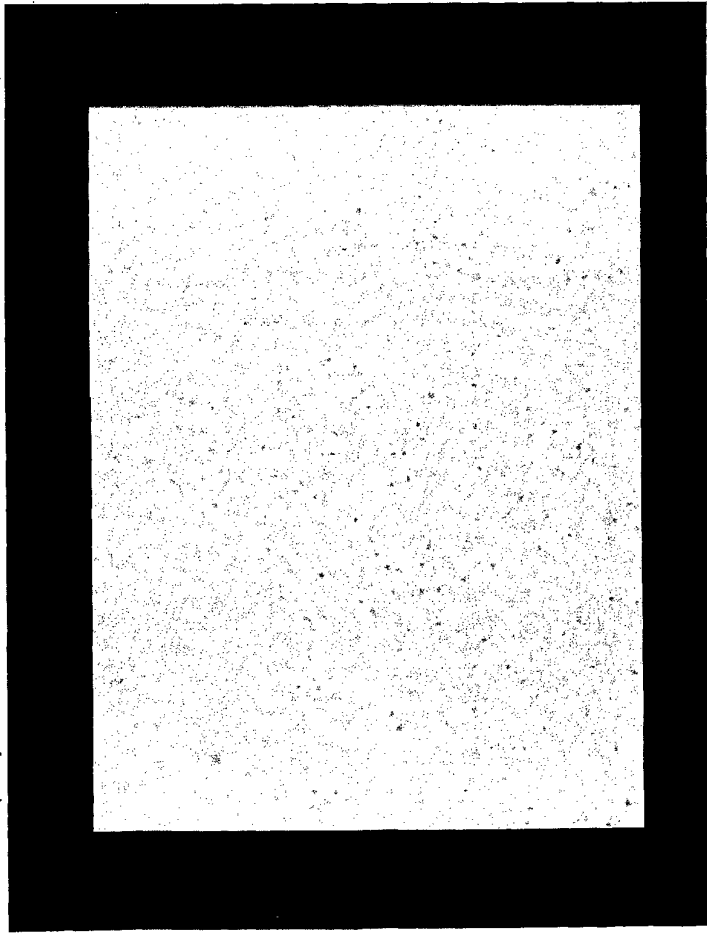
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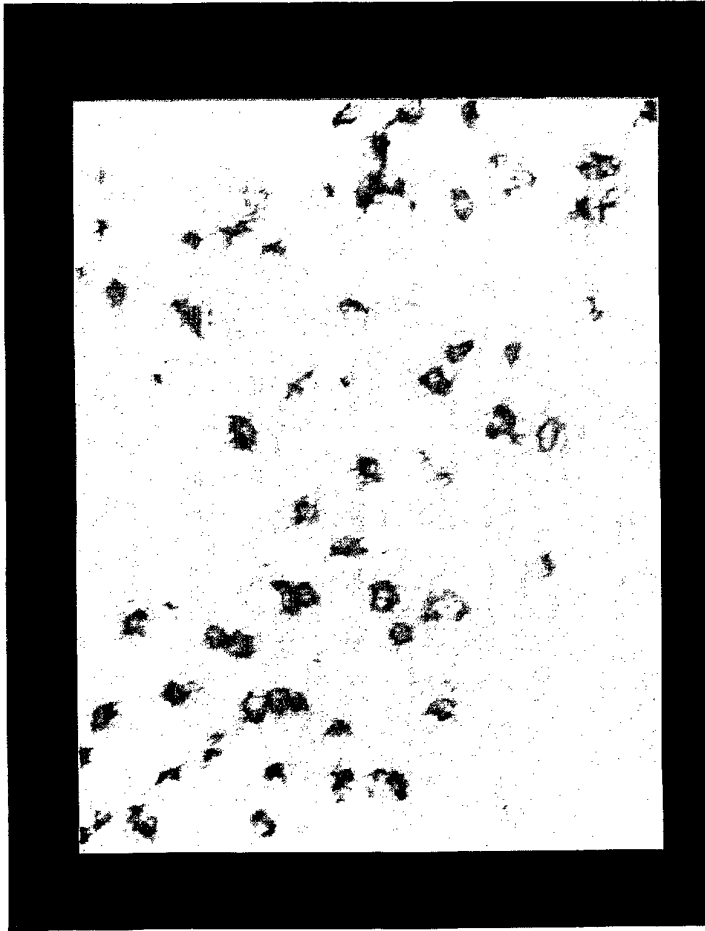
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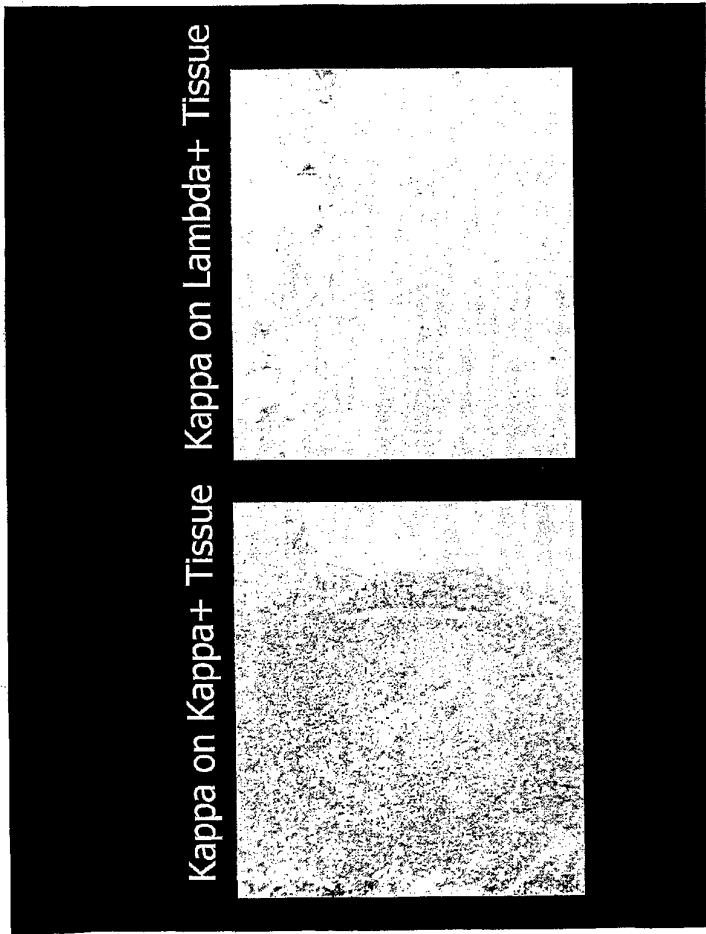




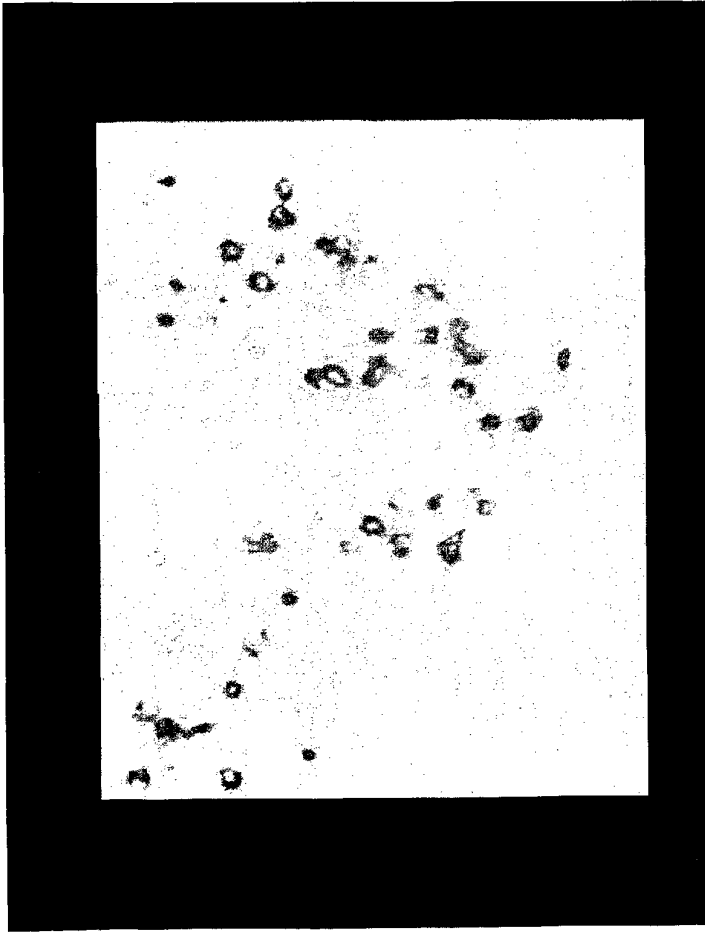


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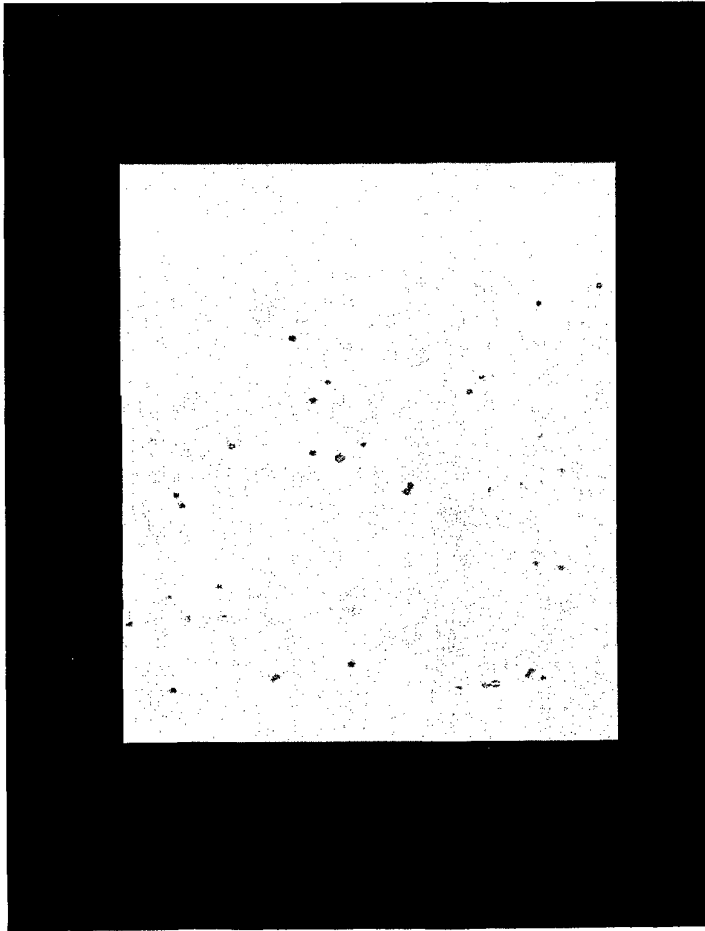




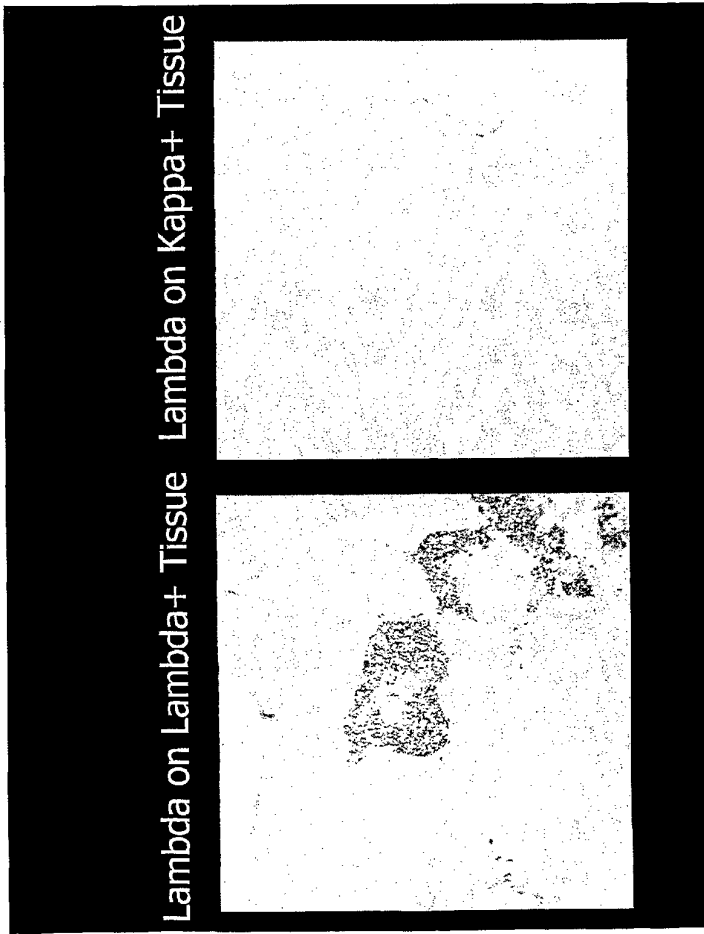
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40



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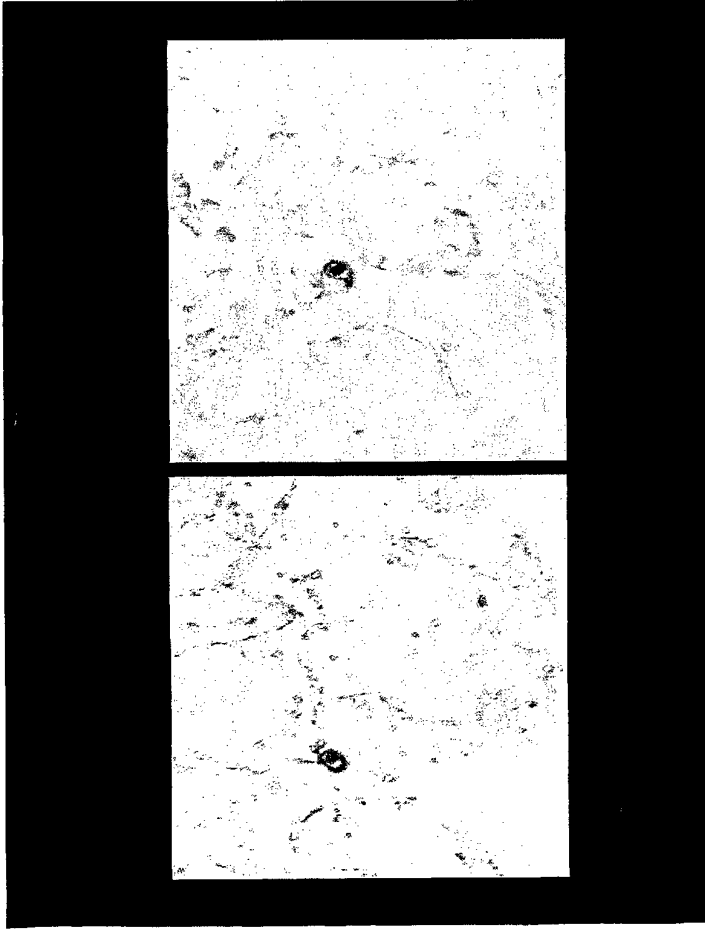


WO 02/22874

19/21

PCT/US01/28014

42

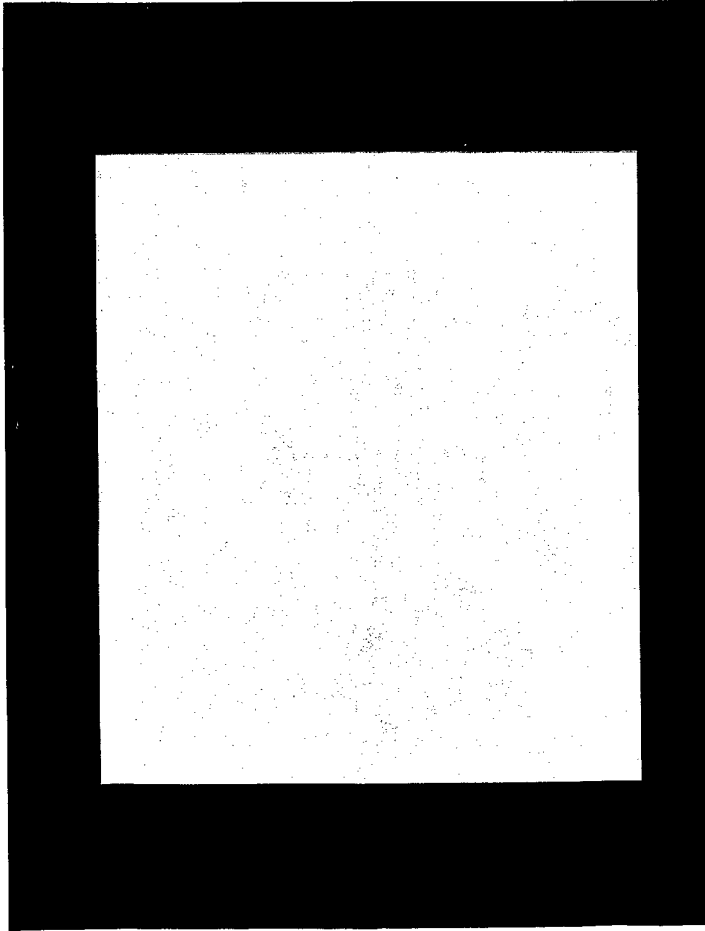


WO 02/22874

20/21

PCT/US01/28014

43

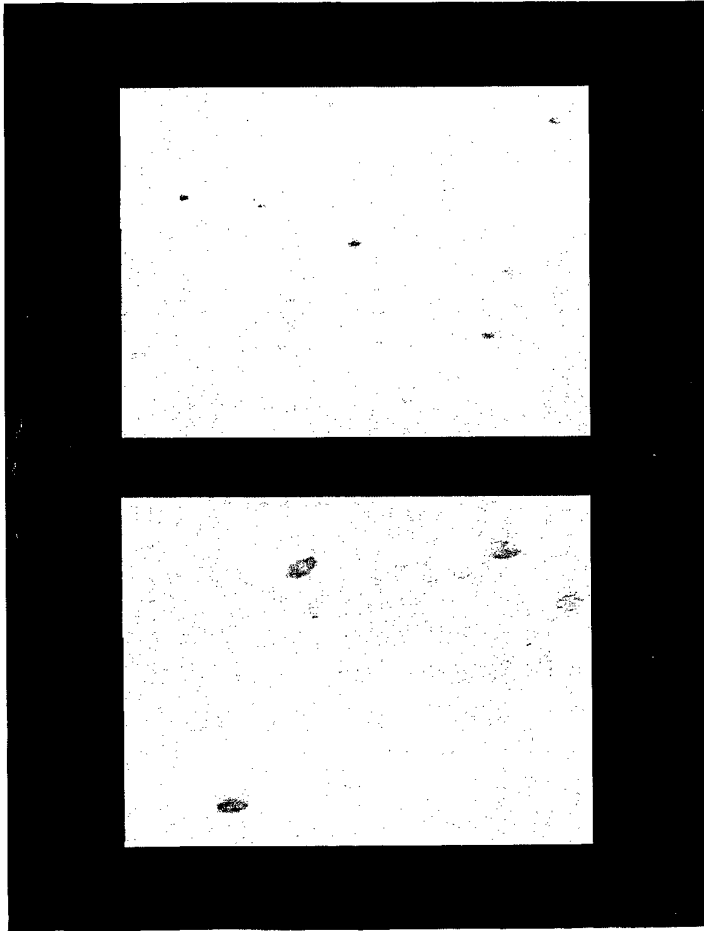


WO 02/22874

21/21

PCT/US01/28014

44



45

WO 02/22874

PCT/US01/28014

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WO 02/22874

PCT/US01/28014

46

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WO 02/22874

PCT/US01/28014

47

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WO 02/22874

PCT/US01/28014

40

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WO 02/22874

PCT/US01/28014

49

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WO 02/22874

PCT/US01/28014

50

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5/

WO 02/22874

PCT/US01/28014

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WO 02/22874

PCT/US01/28014

52

Oligonucleotide probe

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WO 02/22874

PCT/US01/28014

59

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54

WO 02/22874

PCT/US01/28014

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55

WO 02/22874

PCT/US01/28014

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56

WO 02/22874

PCT/US01/28014

Oligonucleotide probe

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WO 02/22874

PCT/US01/28014

57

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58

WO 02/22874

PCT/US01/28014

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WO 02/22874

PCT/US01/28014

59

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WO 02/22874

PCT/US01/28014

Oligonucleotide probe

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61

WO 02/22874

PCT/US01/28014

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      Oligonucleotide probe

<400> 76
agaccagac acggaggcag gctgagtcag           30

<210> 77
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 77
tgttggtcc agtcaggag atggtgatcg           30

<210> 78
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide probe

<400> 78
taaatcatga tttgggggc ttgcctggg           30

<210> 79
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 79
tgttccaga cttggagcca gagaagcgat           30

<210> 80

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62

WO 02/22874

PCT/US01/28014

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<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide probe

<400> 80
aataatcagc ctgctcctca gcctggagcc          30

<210> 81
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide probe

<400> 81
ggtcctccg cggaaaacca cagtgtaact          30

<210> 82
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 82
ttatgagaca caccagtggt gccttgttgg          30

<210> 83
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide probe

<400> 83
ctgctcaggc gtcaggctca gatagctgct          30

<210> 84
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 84

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63

WO 02/22874

PCT/US01/28014

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atgcgtgacc tggcagotgt agcttctgtg          30
<210> 85
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 85
attctgtagg ggccaactgtc ttctccacgg          30
<210> 86
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 86
cctccccctgg gatcctgcag ctctagtctc          30
<210> 87
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 87
tgagggttta ttgagtgcag ggagaagggc          30
<210> 88
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide probe

<400> 88
ggaggtaaaa acagcgtgga tggcgc          25
<210> 89
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:

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64

WO 02/22874

PCT/US01/28014

Oligonucleotide probe

<400> 89  
gaggctggat cggccccgt gtctt 25

<210> 90  
<211> 25  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 90  
aatccgcgtt ccaatgcacc gtcc 25

<210> 91  
<211> 25  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 91  
taaaaactgc gggcaactggg gaagg 25

<210> 92  
<211> 25  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 92  
accgagatt cgcgtggaga tccca 25

<210> 93  
<211> 25  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 93  
gagcaaggag ctgccgagcg accat 25

<210> 94  
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65

WO 02/22874

PCT/US01/28014

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<220>
<223> Description of Artificial Sequence:
      Oligonucleotide probe

<400> 94
acactggtgg tggtagggcat cgtgc                25

<210> 95
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 95
ttccaaatgc gtcaggggtg caagc                25

<210> 96
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 96
agctgcctgc atctttctct gccgc                25

<210> 97
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 97
ccctccacgg ttaacagcac cgcaa                25

<210> 98
<211> 25
<212> DNA
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<220>
<223> Description of Artificial Sequence:
      Oligonucleotide probe

<400> 98
ttggtcacgg gtgtctcggg cctaa                25

<210> 99

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66

WO 02/22874

PCT/US01/28014

<211> 25  
 <212> DNA  
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<220>  
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 Oligonucleotide probe

<400> 99  
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<220>  
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 Oligonucleotide probe

<400> 100  
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<210> 101  
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 <212> DNA  
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<220>  
 <223> Description of Artificial Sequence:  
 Oligonucleotide probe

<400> 101  
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<210> 102  
 <211> 25  
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 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:  
 Oligonucleotide probe

<400> 102  
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<210> 103  
 <211> 25  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:  
 Oligonucleotide probe

<400> 103

67

WO 02/22874

PCT/US01/28014

gtagcggcg cccttgetca catca 25

<210> 104  
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 <212> DNA  
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<210> 105  
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<220>  
 <223> Description of Artificial Sequence:  
 Oligonucleotide probe

<400> 105  
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<210> 106  
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 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:  
 Oligonucleotide probe

<400> 106  
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<210> 107  
 <211> 25  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:  
 Oligonucleotide probe

<400> 107  
 cgggtacagg ggactctggg ggtga 25

<210> 108  
 <211> 25  
 <212> DNA  
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<220>  
 <223> Description of Artificial Sequence:

68

WO 02/22874

PCT/US01/28014

Oligonucleotide probe

<400> 108  
gggtgggtgc tcttgctcc agagg 25

<210> 109  
<211> 25  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 109  
gacctgggt cggtagcacc gcact 25

<210> 110  
<211> 25  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 110  
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<210> 111  
<211> 25  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 111  
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<210> 112  
<211> 26  
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<220>  
<223> Description of Artificial Sequence:  
Oligonucleotide probe

<400> 112  
ggctacagcc acacagbct cctccc 26

<210> 113  
<211> 25  
<212> DNA  
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69

WO 02/22874

PCT/US01/28014

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<220>
<223> Description of Artificial Sequence:
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<400> 113
cgaggcgggc ggatcacctg aggtc          25

<210> 114
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 114
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<210> 115
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 115
ttttttttt ttttttttt ttttttttt          30

<210> 116
<211>
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 116
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<210> 117
<211>
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
      Oligonucleotide probe

<400> 117
CTATTTTCTATTTTCTTTT CGGGAGGCGGAGGTGCGAGTGAGCC          46

<210> 118

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70

WO 02/22874

PCT/US01/28014

<211>  
 <212> DNA  
 <213> Artificial Sequence

<220>  
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 Oligonucleotide probe

<400> 118  
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<210> 119  
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 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:  
 Oligonucleotide probe

<400> 119  
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<210> 120  
 <211>  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:  
 Oligonucleotide probe

<400> 120  
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<210> 121  
 <211>  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:  
 Oligonucleotide probe

<400> 121  
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<210> 122  
 <211>  
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<220>  
 <223> Description of Artificial Sequence:  
 Oligonucleotide probe



## 【 国際調査報告 】

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 01/28014		
A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12Q1/68		
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) IPC 7 C12Q		
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 practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DIRKS R W ET AL: "Methodologies for specific intron and exon RNA localization in cultured cells by haptenized and fluorochromized probes." JOURNAL OF CELL SCIENCE. ENGLAND APR 1993, vol. 104 ( Pt 4), April 1993 (1993-04), pages 1187-1197, XP002226881 ISSN: 0021-9533	
<input type="checkbox"/> Further documents are listed in the continuation of box C.		<input type="checkbox"/> Patent family members are listed in annex.
* Special categories of cited documents:		
*A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed *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 *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 *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *Z* document member of the same patent family		
Date of the actual completion of the international search 10 January 2003	Date of mailing of the international search report 13.05.03	
Name and mailing address of the ISA European Patent Office, P.B. 5618 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Chakravarty, A	

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 01/28014

**Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

- 1.  Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
- 2.  Claims Nos.: 1-6, 8-11  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
  
- 3.  Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

- 1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
- 2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
- 3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
- 4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-11

**Remark on Protest**

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

International Application No. PCT/US 01 28014

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-6,8-11

Present claims 1-6 and 8-11, relate to an extremely large number of possible compounds. Moreover, claim 1 lacks clarity: it is not clear whether the sequence (CTATTT)<sub>n</sub> and the sequence (AAAATAG)<sub>n</sub> should be in the same strand or whether the claim intends to include double stranded molecules.

Consequently, the search has been carried out for those parts of the application which do appear to be clear (and/or concise), namely oligonucleotides exactly identical to SEQ ID 58 (included in the subject-matter of claim 7).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

International Application No. PCT/US 01 28014

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## 1. Claims: 1-11

Any nucleic acid molecule at least containing CTATTT and/or AAAATAG at least once. N.B. this includes the subject-matter of claim 9 because the "second domain" is not defined and can be more or less any sequence.

## 2. Claim : 12

Any nucleic acid sequence containing either CTATTT and/or AAAATAG twice, where these sequences flank another sequence.

## 3. Claims: 13-17

Groups 3-58: each individual SEQ ID as set out in claims 13-17.

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フロントページの続き

(72)発明者 コノーグトン, ジョン

アメリカ合衆国メリーランド州 2 0 8 8 2 レイトンズビル・ハントマスターロード 9 1 1 7

Fターム(参考) 4B024 AA20 CA01 CA09 CA11 HA14 HA19

【要約の続き】

- B a r r RNA) 遺伝子 (配列番号: 5 1 - 5 4 を含めて); A l u (配列番号: 5 5 - 5 6); ポリA (配列番号: 5 7); および検出テール (配列番号: 3 3 0)。

专利名称(译)	用于原位分析的寡核苷酸探针和用于标记蛋白质配方的寡核苷酸序列		
公开(公告)号	<a href="#">JP2005503753A</a>	公开(公告)日	2005-02-10
申请号	JP2002527314	申请日	2001-09-06
[标]申请(专利权)人(译)	文塔纳医疗系统公司		
申请(专利权)人(译)	每次塔纳Medeikaru系统公司的Rete		
[标]发明人	ウターモーレンジヨセフジー コノーグトンジヨン		
发明人	ウターモーレン,ジヨセフ・ジー コノーグトン,ジヨン		
IPC分类号	G01N33/53 C12N15/09 C12Q1/68 C12Q1/6816 C12Q1/6841 C12Q1/6876 C12Q1/6888 G01N33/532 G01N33/569		
CPC分类号	C12Q1/6841 C12Q1/6811 C12Q1/6816 C12Q1/6876 C12Q1/6888 C12Q2600/158 C12Q2525/161 C12Q2525/151 C12Q2537/143		
FI分类号	C12N15/00.ZNA.A G01N33/53.M G01N33/532.B G01N33/569.L		
F-TERM分类号	4B024/AA20 4B024/CA01 4B024/CA09 4B024/CA11 4B024/HA14 4B024/HA19		
优先权	60/233177 2000-09-15 US		
其他公开文献	JP2005503753A5 JP4986367B2		
外部链接	<a href="#">Espacenet</a>		

摘要(译)

本发明提供寡核苷酸探针和寡核苷酸探针集合和蛋白质标记，用于检测或定位细胞或组织样品中的多个核酸靶基因或抗原。具体而言，本发明是，每个探针序列式 (CTATTTT) 名词，(AAAATAG) 名词，或 (TTTTATC) 名词或 (GATAAAA) 名词 [其中，所有当“n”是等于或超过在1]一种用于在具有与标签域的情况下的原位杂交分析中使用寡核苷酸探针。本发明提供寡核苷酸探针的集合或“混合物”，用于检测或定位细胞或组织样品中的特定核酸靶基因。鸡尾酒是用于检测以下：卡帕基因 (SEQ ID NO : 1-16, 其中包括) 有用;拉姆达基因 (SEQ ID NO : 501-509,511-513, 和515); CMV (巨细胞病毒) 基因 (SEQ ID NO : 221-241包括); EBER (爱泼斯坦 - BarrRNA) 基因 (SEQ ID NO : 51-54, 舍); 铝 (SEQ ID NO : 55-56); 聚一 (SEQ ID NO : 57); 和检测尾 (SEQ ID NO : 330)。

