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 (71) Demandeur/Applicant:
 VIRCO BVBA, BE
 (72) Inventeurs/Inventors:
 LARDER, BRENDAN, GB;
 KEMP, SHARON, GB;
 BLOOR, STUART, GB;
 BROPHY, ANN, GB
 (74) Agent: GOWLING LAFLEUR HENDERSON LLP

(54) Titre : PROCEDE DE DETECTION DE MUTATION DANS DES HIV AU MOYEN DE SEQUENCAGE POL
 (54) Title: METHOD FOR MUTATION DETECTION IN HIV USING POL SEQUENCING

(57) **Abrégé/Abstract:**

The present invention relates to a method for mutation analysis of the HIV pol gene of HIV virions comprising amplifying virion RNA or DNA via nested PCR using outer primers as represented in SEQ ID No. 1 and 2, amplifying said PCR product via nested PCR using a 5' and 3' primer chosen from the inner primers SEQ ID No. 3, 4, 5, and 6, and sequencing this secondary obtained PCR product using at least one sequencing primer chosen from any of SEQ ID No. 7 to 12 or variants thereof. In the alternative, at least one secondary sequencing primer may be used chosen from any of SEQ ID No. 13 to 24. The benefit of the sequences present in the invention resides in the fact that, with the aid of the oligonucleotides, the sequences of all presently known HIV subtypes and all mutations of the pol gene presently known to yield resistance towards antiretroviral therapy can be determined. The present invention also relates to kits for performing such a method as well as primers for performing the same.

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- (71) Applicant (for all designated States except US): **VIRCO N.V.** [BE/BE]; Generaal De Wittelaan L11B4, B-2800 Mechelen (BE).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **LARDER, Brendan** [GB/GB]; Monona 6, Churchlane, Cambridge, Cambridgeshire CB3 7HQ (GB). **KEMP, Sharon** [GB/GB]; Monona 6, Churchlane, Cambridge, Cambridgeshire CB3 7HQ (GB). **BLOOR, Stuart** [GB/GB]; 14 Coltsfoot, Biggleswade, Bedfordshire SG18 8SR (GB). **BROPHY, Ann** [GB/GB]; 114 Sleaford Street, Cambridge, Cambridgeshire CB1 2NS (GB).
- (74) Agent: **DAELEMANS, Frank**; Tibotec N.V., Intellectual Property Dept., Generaal De Wittelaan L11B3, B-2800 Mechelen (BE).
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(54) Title: METHOD FOR MUTATION DETECTION IN HIV USING POL SEQUENCING

(57) Abstract: The present invention relates to a method for mutation analysis of the HIV pol gene of HIV virions comprising amplifying virion RNA or DNA via nested PCR using outer primers as represented in SEQ ID No. 1 and 2, amplifying said PCR product via nested PCR using a 5' and 3' primer chosen from the inner primers SEQ ID No. 3, 4, 5, and 6, and sequencing this secondary obtained PCR product using at least one sequencing primer chosen from any of SEQ ID No. 7 to 12 or variants thereof. In the alternative, at least one secondary sequencing primer may be used chosen from any of SEQ ID No. 13 to 24. The benefit of the sequences present in the invention resides in the fact that, with the aid of the oligonucleotides, the sequences of all presently known HIV subtypes and all mutations of the pol gene presently known to yield resistance towards antiretroviral therapy can be determined. The present invention also relates to kits for performing such a method as well as primers for performing the same.



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METHOD FOR MUTATION DETECTION IN HIV USING POL SEQUENCING

This application claims priority of US. Patent Application No. 09/640,787 filed August
5 18th 2000 and EP Patent Application No. 00201433.0 filed April 18th 2000, the contents
of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to a method for detecting mutations within the
10 HIV pol gene of HIV isolates and in particular with the design of amplification primers
and sequencing primers for use in the analysis of the coding domains for the protease
and reverse transcriptase, respectively.

BACKGROUND OF THE INVENTION

15 The rapid and specific detection of infectious agents such as HIV is of utmost
importance both for the diagnosis of the infection as well as to monitor the therapy of
the infected patients. In order to reduce the analytical window period, sequence based
approaches are increasingly used. Detection methods based on hybridization suffer
from reduced reliability because of the huge viral mutagenicity. Therefor sequencing
20 based methods are very much desired as tools to interrogate the particular viral
sequence of a biological sample.

The availability of rapid, high-throughput automated DNA sequencing
technology has obvious applications in clinical research, including the detection of
25 variations in virus populations and mutations responsible for drug resistance in virus
genomes. However, analysis of clinical samples by manual sequencing or polymerase
chain reaction-(PCR) based point mutation assays has revealed that complex mixtures
of wild type and mutant HIV genomes can occur during drug therapy. Therefore, to
assess the likely susceptibility of a virus population to a particular drug therapy, it
30 would be desirable to perform DNA sequence analysis that can simultaneously
quantitate several resistance mutations in multiple genomes. A particular advantage of
analysing the sequence of more than one pol gene enzyme (Protease and Reverse
transcriptase) is that the studied material reflects to a greater extent the viral genetic
diversity in the particular patient being investigated.

35

The main target cell for HIV infection was identified as the CD4+ subset of
T-cells. In order to replicate, HIV first interacts with cells expressing the CD4 surface
protein and co-receptor via binding through the gp120 envelope protein. Following

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fusion via the gp41 domain of the envelope, entry is achieved, the viral particle degraded and the RNA genome transcribed into double-stranded complementary DNA (cDNA). This genetic material is transported into the cell nucleus as part of the pre-integration complex, where the DNA is processed by viral integrase and incorporated
5 into the host genome. In an activated cell, the viral genome is transcribed and subsequently translated into structural proteins and enzyme precursors. The polyproteins, Gag and Gag-Pol containing matrix, capsid, nucleocapsid as well as the enzymes reverse transcriptase, protease and integrase are directed to the cell membrane where proteolytic cleavage by viral protease and virion packaging occurs. Most of
10 these events have been extensively studied and a number of stages for possible intervention to prevent viral replication have been identified. These include attachment and entry into the host cell, formation of proviral DNA by reverse transcriptase enzymes, integration of proviral DNA into the host cell chromosomes by integrase, as well as virus assembly, including cleavage of the precursor viral proteins, by viral
15 protease. Clinically relevant agents have been developed against two of the viral genes, reverse transcription and protease.

The efficacy of these compounds is largely depending on the mutations present in these proteins. HIV has no proofreading mechanisms and therefore has a high
20 mutagenic power. This high mutagenic capacity enables the virus to induce resistance to the therapy by the introduction of mutations in those genes.

Retroviral inhibitors may block viral replication in various ways. For example, Nucleoside Reverse Transcriptase Inhibitors (NRTIs), compete with the natural
25 nucleoside triphosphates for incorporation into elongating viral DNA by reverse transcriptase. Chemical modifications that distinguish these compounds from natural nucleosides result in DNA chain termination events. NRTIs that are currently available include for instance zidovudine (ZDV), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC) and abacavir (ABC).
30

Nucleotide reverse transcriptase inhibitors (NtRTIs) have the same mode of action as NRTIs, but they differ in that they are already monophosphorylated and therefore they require fewer metabolic steps. For example Adefovir (bis-POM-PMEA) and bis-POC PMPA belong to this category of treatments.
35

Non-Nucleoside Reverse Transcriptase inhibitor (NNRTIs) are a group of structurally diverse compounds which inhibit HIV reverse transcriptase by noncompetitive binding to or close to the active site of the viral reverse transcriptase

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enzyme, thereby inhibiting its activity. Available compounds in this group include for instance nevirapine (NVP), delavirdine (DLV) and efavirenz.

5 Protease Inhibitors (PIs) are peptidomimetic and bind to the active site of the viral protease enzyme, thereby inhibiting the cleavage of precursor polyproteins necessary to produce the structural and enzymatic components of infectious virions. PIs that are currently available include for instance saquinavir (SQV), ritonavir (RTV), indinavir (IDV) nelfinavir (NFV), amprenavir (APV) and lopinavir (ABT-378).

10 The options for antiretroviral therapy have improved considerably as new agents have become available. Current guidelines for antiretroviral therapy recommend a triple combination therapy regimen for initial treatment, such as one PI and 2 NRTIs or one NNRTI and 2 NRTIs. These combination regimens show potent antiretroviral activity and are referred to as HAART (highly active antiviral therapy). The introduction of
15 HAART has resulted in a significant reduction of morbidity and mortality in HIV-1 patient populations with access to these drugs .

Assays for detection of mutations in HIV-1 are based on polymerase chain reaction (PCR) amplification of viral genomic sequences. These amplified sequences
20 are then analyzed using either hybridization or sequencing techniques. Hybridization-based assays include primer-specific PCR, which makes use of synthetic oligonucleotides designed to allow selective priming of DNA synthesis. See Larder, B.A., et al., AIDS 5, 137-144 (1991); Richman, D.D., et al., J. Infect. Dis. 164, 1075-1081 (1991); Gingeras, T.R., et al., J. Infect. Dis. 164, 1066-1074 (1991). Only when
25 primer sequences match the target sequence (wild-type or mutant) at the 3' end, is amplification of target sequences possible and DNA fragments are produced. Knowledge of the primer sequences allows one to infer the sequence of the viral isolate under investigation, but only for the region covered by the primer sequences. Other hybridization-based assays include differential hybridization (Eastman, P.S., et al., J. Acq. Imm. Def. Syndr. Human Retrovirol. 9, 264-273 (1995); Holodniy, M., et al., J. Virol. 69, 3510-3516 (1995); Eastman, P.S., et al., J. Clin. Micro. 33, 2777-2780(1995).); Line Probe Assay (LiPA® HIV-11 RT, Innogenetics) (Stuyver, L., et al., Antimicrob. Agents Chemotherap. 41, 284-291 (1997).); Oligonucleotide ligation assay (Edelstein, R. et al. J. Clin Microbiol. 36(2), 569-572 (1998)) and GeneChip
35 technology (Affymetrix) (D'Aquila, R.T. Clin. Diagnost. Virol. 3, 299-316 (1995); Fodor, S.P.A. et al., Nature 364, 555-556 (1993); Fodor, S.P.A. Nature 227, 393-395 (1997). DNA sequencing assays provide information on all nucleotides of the sequenced region. Target sequences are amplified by PCR. Sequence analysis is

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primarily based on the incorporation of dideoxy chain-terminating nucleotides (lacking 3' hydroxyl groups) in elongating DNA sequences and gel-electrophoretic analysis of the resulting molecules. Sequencing technologies can be semi-automated and make use of fluorescently labeled primers or ddNTPs to "read" off the sequence from a
5 polyacrylamide gel. Novel techniques and approaches to determine mutations are being developed and are evenly well suited to determine mutations present in a sample under investigation. Other assays to determine mutations have become available *e.g.* Invader® assay (Third Wave Technologies, Inc.), WAVE® DNA assay (Transgenomic, Inc.), mass spectrometry (Jackson P., et al. *Molecular Medicine Today* 6, 271-276, (2000)) and surface plasmon resonance (Nakatani, K. et al. *Nature Biotechnology* 19(1), 18-19, (2001). An overview of currently used mutation techniques, comprising gel based and non-gel based analyses are surveyed in Shi, M. *Clin. Chem.* 2001, (47:2) 164-172. Sequence analysis may be performed on either
10 nucleic acid material not limited to DNA and RNA.

15

Viruses devoid of proofreading mechanisms have a high mutagenic power. This mutagenic capacity provides the infectious agent with a means to escape drug treatment, by changing the drug targets. This leads to reduced drug efficacy, resistance and thus increased patient morbidity and mortality. One approach to detect the viral
20 resistance towards pharmacological treatment involves the determination of those mutations occurring in the viral genome. In order to determine these mutations several approaches are available. Hybridization based methods (differential hybridization, BioChips, LiPa®, primer specific PCR) have been developed, however, these methods suffer from the disadvantage that only a limited set of mutations can be screened per
25 analytical run.

Alternatively, sequencing methods have been developed. Although this technology increases reliability when compared to hybridization methods, the current protocols do not allow to reliably and within an acceptable analytical window period
30 sequence a gene such as the HIV pol gene with all its mutations which may occur during viral mutagenesis under treatment pressure. Therefore the diagnostic value of existing sequencing methods is limited whereas the need for fast, reliable and complete sequence analysis methods is high in the field of HIV diagnostics.

35

The present invention concerns an improved sequencing method involving a set of primers providing a means to amplify and sequence the pol gene comprising all mutations. In addition, the present method also allows the analysis of mixed samples. The primer combination of the present invention reduces the analytical period since all

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mutations can be sequenced in a single laboratory format, avoiding the necessary step of additional cloning or resequencing part of the viral genome in order to identify all mutations related to drug resistance. Resequencing of the genome becomes necessary when due to viral mutagenesis, a defined primer does not hybridize properly to its target sequence. This delays the laboratory turnaround time. Using the protocol of the present invention the sequence of the sample is reliably determined on a single day. Therefore the method and the primer combination of the present invention improve the monitoring of drug resistance, leading to an improved patient management.

10 The aim of the present invention is thus to provide a reliable sequence analysis method and kit for performing mutation analysis of the pol gene of HIV virus isolates.

The pol gene of HIV codes for different proteins including protease, reverse transcriptase, integrase.

15

The present invention relates to a method for mutation analysis of the HIV pol gene of a HIV virion comprising the steps of:

- a) isolation of a sample,
- 20 b) virion RNA extraction of the isolated sample material,
- c) amplifying RNA via nested PCR using outer primers as represented in SEQ ID No. 1 (OUT3) and 2 (PRTO-5),
- d) amplifying said PCR product via nested PCR using a 5' and 3' primer chosen from the inner primers as represented in SEQ ID No. 3 (PCR2.5), 4 (PCR2.3), 5 (SK107) and 6 (SK108), and
- 25 e) sequencing this secondary obtained PCR product using at least one sequencing primer chosen from any of SEQ ID No. 7 to 12 (Seq1FOR, Seq2FOR, Seq3F, Seq1B, Seq3B, Seq6R, Seq1F, Seq2A, Seq3A, Seq5A, Seq7A, Seq2B, Seq4B, Seq6B, Seq7B, Seq4A, Seq6A, Seq5B; see Table 1).

30

The present invention describes a mutation analysis of the pol gene of HIV. It should be appreciated that the group of HIV viruses contains several families HIV-1 and HIV-2. HIV-1 is present throughout the world whereas HIV-2 is widespread in West-Africa. HIV-1 isolates including group M and group O viruses, in particular group M viruses. Mixed populations carrying mutations can be detected when present down to at least 20%.

35

The present invention also provides a method for mutation analysis of the HIV pol gene of HIV isolates comprising the steps of:

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- a) isolation of a sample,
- b) viral DNA extraction of the isolated sample material,
- c) amplifying DNA via nested PCR using outer primers as represented in SEQ ID No. 1 (OUT3) and 2 (PRTO-5),
- 5 d) amplifying said PCR product via nested PCR using a 5' and 3' primer chosen from the inner primers as represented in SEQ ID No. 3 (PCR2.5), 4 (PCR2.3), 5 (SK107) and 6 (SK108), and
- e) sequencing this secondary obtained PCR product using at least one sequencing primer chosen from any of SEQ ID No. 7 to 12 (Seq1FOR, Seq2FOR, Seq3F, Seq1B, Seq3B, Seq6R, Seq1F, Seq2A, Seq3A, Seq5A, Seq7A, Seq2B, Seq4B, Seq6B, Seq7B, Seq4A, Seq6A, Seq5B; see Table 1).

15 According to a preferred method said secondary PCR product is sequenced using a primer as represented in SEQ ID No. 7 (Seq1FOR).

According to a preferred method said secondary PCR product is sequenced using a primer as represented in SEQ ID No. 8 (Seq2FOR).

20 According to a preferred method said secondary PCR product is sequenced using a primer as represented in SEQ ID No. 9 (Seq3F).

According to a preferred method said secondary PCR product is sequenced using a primer as represented in SEQ ID No. 10 (Seq1B).

25 According to a preferred method said secondary PCR product is sequenced using a primer as represented in SEQ ID No. 11 (Seq3B).

30 According to a preferred method said secondary PCR product is sequenced using a primer as represented in SEQ ID No. 12 (Seq6R).

The present invention also provides a method according to the present invention wherein one of the initial sequencing primers is replaced by one or a pair of replacement primers (Table 2). For example, if Seq2FOR (SEQ ID No. 8) failed it is replaced by Seq3A (SEQ ID No. 15) and Seq5A (SEQ ID No. 16). However in principle any described primer that obtains sequence from the region that Seq2FOR (SEQ ID No. 8) was expected to cover can be used i.e. Seq3A (SEQ ID No. 15), Seq4A (SEQ ID No. 22) or Seq5A (SEQ ID No. 16) (see Figure 1). In addition, Seq6A (SEQ ID No.23) and Seq5B (SEQ ID No. 24) were also not proposed to replace a specific initial primer but can be used to cover respective sequence domains (see Figure 1).

In preferred methods according to the present invention the initial sequencing primer as represented in SEQ ID No 7 (Seq1FOR) is replaced by a primer set as represented in SEQ ID No. 13 (Seq1F) and 14 (Seq2A).

5

In preferred methods according to the present invention the initial sequencing primer as represented in SEQ ID No 8 (Seq2FOR) is replaced by a primer set as represented in SEQ ID No. 15 (Seq3A) and 16 (Seq5A).

10

In preferred methods according to the present invention the initial sequencing primer as represented in SEQ ID No 9 (Seq3F) is replaced by a primer set as represented in SEQ ID No. 16 (Seq5A) and 17 (Seq7A).

15

In preferred methods according to the present invention the initial sequencing primer as represented in SEQ ID No 10 (Seq1B) is replaced by a primer set as represented in SEQ ID No. 4 (PCR2.3) and 18 (Seq2B).

20

In preferred methods according to the present invention the initial sequencing primer as represented in SEQ ID No 11 (Seq3B) is replaced by a primer set as represented in SEQ ID No. 18 (Seq2B) and 19 (Seq4B).

25

In preferred methods according to the present invention the initial sequencing primer as represented in SEQ ID No 12 (Seq6R) is replaced by a primer set as represented in SEQ ID No. 20 (Seq6B) and 21 (Seq7B).

30

Preferably, the methods according to present invention involve a sequencing step wherein said secondary PCR product is sequenced using a primer as represented in SEQ ID No 13 (Seq1F).

35

Preferably, the methods according to present invention involve a sequencing step wherein said secondary PCR product is sequenced using a primer as represented in SEQ ID No 14 (Seq2A).

40

Preferably, the methods according to present invention involve a sequencing step wherein said secondary PCR product is sequenced using a primer as represented in SEQ ID No 15 (Seq3A).

45

Preferably, the methods according to present invention involve a sequencing step wherein said secondary PCR product is sequenced using a primer as represented in SEQ ID No 16 (Seq5A).

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Preferably, the methods according to present invention involve a sequencing step wherein said secondary PCR product is sequenced using a primer as represented in SEQ ID No 17 (Seq7A).

5 Preferably, the methods according to present invention involve a sequencing step wherein said secondary PCR product is sequenced using a primer as represented in SEQ ID No 18 (Seq2B).

10 Preferably, the methods according to present invention involve a sequencing step wherein said secondary PCR product is sequenced using a primer as represented in SEQ ID No 19 (Seq4B).

15 Preferably, the methods according to present invention involve a sequencing step wherein said secondary PCR product is sequenced using a primer as represented in SEQ ID No 20 (Seq6B).

20 Preferably, the methods according to present invention involve a sequencing step wherein said secondary PCR product is sequenced using a primer as represented in SEQ ID No 21 (Seq7B).

Preferably, the methods according to present invention involve a sequencing step wherein said secondary PCR product is sequenced using a primer as represented in SEQ ID No 22 (Seq4A).

25 Preferably, the methods according to present invention involve a sequencing step wherein said secondary PCR product is sequenced using a primer as represented in SEQ ID No 23 (Seq6A).

30 Preferably, the methods according to present invention involve a sequencing step wherein said secondary PCR product is sequenced using a primer as represented in SEQ ID No 24 (Seq5B).

35 The invention further relates to primers having at least 80% sequence similarity to the sequences represented in SEQ ID 1-24, preferably at least 90% sequence similarity to the sequences represented in SEQ ID 1-24, more preferably at least 95% sequence similarity to the sequences represented in SEQ ID 1-24

40 The invention further relates to primers comprising at least 8 consecutive nucleotides, wherein said sequence of at least 8 consecutive nucleotides is present in SEQ ID No. 1-24

A primer acts as a point of initiation for synthesis of a primer extension product that is complementary to the nucleic acid strand to be copied. The place of hybridization is determined by the primer- and target sequence. As known by the skilled person in the art, specificity of the annealing can be guaranteed by choosing a sequence domain within the target sequence, which is unique, compared to other non-target sequences. Nevertheless, start and stop of the primer onto the target sequence may be located some nucleotides up- or downstream the defined primer site without interfering with this specificity.

10

Consequently, the present invention also provides a method as described above wherein the sequencing primer is chosen up to 1, 2, 3 or 4 nucleotides upstream or downstream the described primer region.

The present invention also provides a method as described above wherein the outer primer is chosen up to 1, 2, 3 or 4 nucleotides upstream or downstream the described primer region.

The present invention also provides a method as described above wherein the inner primer is chosen up to 1, 2, 3 or 4 nucleotides upstream or downstream the described primer region.

The present invention also provides a method as described above wherein the sample contains free virion particles or virus infected cells.

25

In particular, the present invention also provides a method as described above wherein the sample is any biological material taken either directly from the infected human being (or animal), or after culturing (*e.g.* for enrichment). Biological material may be *e.g.* expectorations of any kind, broncheolavages, blood (plasma, serum), skin tissue, biopsies, sperm, semen, lymphocyte blood culture material, colonies, liquid cultures, faecal samples, urine etc.

30

In one embodiment of the present invention, a biological sample is taken of a human being or animal treated or being treated with antiretroviral drug regimens.

35

The present invention also relates to a primer as described above (see Table 1) and used to analyse the sequence of the HIV pol gene of HIV isolates.

Preferentially, such methods according to the present invention involve the sequencing of the defined primary PCR product.

40

In an embodiment the present invention relates to a method as described above, wherein the mutation identified confers resistance to an antiretroviral drug.

5 In a further embodiment the present invention relates to a method as described above, wherein the mutation identified confers resistance to a protease inhibitor.

In one embodiment the present invention relates to a method as described above, wherein the mutation identified confers resistance to a reverse transcriptase inhibitor.

10

In one embodiment the present invention relates to a method as described above, wherein the mutation identified confers resistance to an integrase inhibitor.

The present invention also relates to a diagnostic kit for the mutation analysis of the HIV pol gene of HIV-1 isolates comprising at least one of the primers as shown in Table 1. The following definitions serve to illustrate the terms and expressions used in the present invention.

The term "drug-induced mutation" means any mutation different from consensus wild-type sequence, more in particular it refers to a mutation in the HIV protease or RT coding region that, alone or in combination with other mutations, confers a reduced susceptibility of the isolate to the respective drug.

The term "target sequence" as referred to in the present invention describes the nucleotide sequence of the wild type, polymorphic or drug induced variant sequence of the protease and RT gene of HIV-1 isolates to be specifically detected by sequence analysis according to the present invention. This nucleotide sequence may encompass one or several nucleotide changes. Target sequences may refer to single nucleotide positions, nucleotides encoding amino acids or to sequence spanning any of the foregoing nucleotide positions. In the present invention said sequence often includes one or two variable nucleotide positions. Sequence alterations detected by the present method include but are not limited to single nucleotide mutations, substitutions, deletions, insertions, inversions, repeats or variations covering multiple variations, optionally present at different locations. Sequence alterations may further relate to epigenetic sequence variations not limited to for instance methylation. Sequence analysis can be performed both on all types of nucleic acid including RNA and DNA.

35

It is to be understood that the complement of said target sequence is also a suitable target sequence in some cases.

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The target material in the samples to be analysed may either be DNA or RNA, *e.g.* genomic DNA, messenger RNA, viral RNA, proviral nucleic acid or amplified versions thereof. These molecules are also termed polynucleic acids. It is possible to use DNA or RNA molecules from HIV samples in the methods according to the present invention.

Well-known extraction and purification procedures are available for the isolation of RNA or DNA from a sample (*e.g.* in Maniatis et al., *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press (1989)).

The term "primer" refers to single stranded sequence-specific oligonucleotide capable of acting as a point of initiation for synthesis of a primer extension product that is complementary to the nucleic acid strand to be copied. The length and the sequence of the primer must be such that they allow priming the synthesis of the extension products.

Preferentially, the primer is about 5-50 nucleotides long. Specific length and sequence will depend on the complexity of the required DNA or RNA targets, as well on the conditions of primer use such as temperature and ionic strength.

The one skilled in the art will know that the primers of the present invention can be replaced by their complementary strands.

The fact that amplification primers do not have to match exactly with the corresponding template to warrant proper amplification is ample documented in the literature (Kwok et al. 1990).

The primers of the present invention also comprise those oligonucleotides having at least 80% similarity to the sequences in SEQ ID 1-24, preferentially at least 90% and more preferentially at least 95% similarity according to the FASTA or BLAST algorithms. (Altschul et al. "Basic local alignment search tool *J. Mol. Biol.* 1990, 215, 403-410, <http://www.ncbi.nlm.nih.gov/blast>; Lipman et al. "Rapid and sensitive protein similarity searches. *Science* 1985, 227, 1435-1441. <http://www.ebi.ac.uk>)

A "sequence similar to" a DNA sequence is not limited to any particular sequence, but is defined as such a sequence modified with substitutions, insertions, deletions, and the like known to those skilled in the art so that the function or activity of its encoded protein is substantially at the same level. Herein, "similarity" is defined as the rate (%) of identical nucleotides within a similar sequence with respect to a

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reference sequence. Similarity is an observable quantity that might be expressed as, for example, % identity, wherein identity means identical nucleotides. Homology refers to a conclusion drawn from these data.

5 Oligonucleotide generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, oligonucleotides as used herein refer to, single-stranded DNA, or single-stranded RNA. As used herein, the term oligonucleotide includes DNAs or RNAs as described above that contain one or more modified bases. Thus, 10 DNAs or RNAs with backbones modified for stability or for other reasons are "oligonucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are oligonucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that 15 serve many useful purposes known to those of skill in the art. The term oligonucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of oligonucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells, inter alia. Polynucleotides embraces short polynucleotides often referred to as oligonucleotide(s). 20

There are several methods reported for amplifying nucleic acids. These methods comprise cycling techniques, isothermal reactions and combinations thereof. The amplification method used can be either polymerase chain reaction (PCR; Saiki et al. 1988), ligase chain reaction (LCR; Landgren et al. 1988; Wu and Wallace 1989; 25 Barany 1991), nucleic acid sequence-based amplification (NASBA; Guatelli et al. 1990; Compton 1991), transcription-based amplification system (TAS; Kwoh et al. 1989), strand displacement amplification (SDA; Duck 1990; Walker et al. 1992), rolling circle amplification (Lizardi, 1998, Zhang 1998, "Circular probe amplification using energy-transfer primers" provisional application filed) or amplification by means 30 of Qss replicase (Lizardi et al. 1988; Lomeli et al. 1989) or any other suitable method to amplify nucleic acid molecules known in the art.

The oligonucleotides used as primer may also comprise nucleotide analogues such as phosphothiates (Matsukura et al. 1987), alkylphosphorothiates (Miller et al. 35 1979) or peptide nucleic acids (Nielsen et al. 1991; Nielsen et al. 1993) or may contain intercalating agents (Asseline et al. 1984).

The oligonucleotides used as primer in the sequencing reaction may also

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contain labels. These labels comprise but are not limited to radionucleides, fluorescent labels, biotin, chemiluminescent labels.

The oligonucleotides of the present invention may be labelled by groups
 5 enabling the capture of the amplified fragment *e.g.* biotin. These capture ligands enable both the detection of the nucleotides or the amplified fragment containing them and the recovery of the oligonucleotides or the amplified fragment containing them from complex mixtures.

10 The nucleotides used in the present invention may also be substituted by *e.g.* biotin, fluorescent labels or radionucleides or may contain unnatural bases.

The oligonucleotides used for the present invention can be used for the different sequencing technologies known in the art, for instance dideoxysequencing, cycle
 15 sequencing, minisequencing and any variants thereof.

Figures and Tables

The figures, tables and examples as given below exemplify the present
 20 invention. These data are not meant to limit the scope of the present invention.

Figure 1 : Schematic overview of the total coding region of the protease- RT coding domain of HIV-1 isolates. The protease domain is shown by a black box, the RT coding region by a shaded box. The length in nucleotides of both coding regions is
 25 indicated. Regions that are sequenced using respectively mentioned sequencing primers are shown. Primary sequences and the secondary sequences are schematically presented.

Table 1 : Sequence of the amplification- and sequencing primers used. Name and
 30 sequence identification numbers are indicated.

NAME	SEQUENCE	SEQ ID N ^o
cDNA synthesis and first round PCR		
OUT3	5'-CAT-TGC-TCT-CCA-ATT-ACT-GTG-ATA-TTT-CTC-ATG-3'	SEQ ID 1
PRTO-5	5'GCC-CCT-AGG-AAA-AAG-GGC-TGT-TGG-3'	SEQ ID 2
Second round (nested) PCR		
Set A		
PCR2.5	5'-CCT-AGG-AAA-AAG-GGC-TGT-TGG-AAA-TGT-GG-3'	SEQ ID 3

PCR2.3	5'-CTA-ACT-GGT-ACC-ATA-ATT-TCA-CTA-AGG-GAG-G-3'	SEQ ID 4
Set B		
SK107	5'-CAT-CTA-CAT-AGA-AAG-TTT-CTG-CTC-C-3'	SEQ ID 5
SK108	5'-CTA-GGA-AAA-AGG-GCT-GTT-GGA-AAT-G-3'	SEQ ID 6
Primary Sequencing primers		
Seq1FOR	5'-GAG-AGC-TTC-AGG-TTT-GGG-G-3'	SEQ ID 7
Seq2FOR	5'-AAT-TGG-GCC-TGA-AAA-TCC-3'	SEQ ID 8
Seq3F	5'-CCT-CCA-TTC-CTT-TGG-ATG-GG-3'	SEQ ID 9
Seq1B	5'-CTC-CCA-CTC-AGG-AAT-CC-3'	SEQ ID 10
Seq3B	5'-GTA-CTG-TCC-ATT-TAT-CAG-G-3'	SEQ ID 11
Seq6R	5'-CTT-CCC-AGA-AGT-CTT-GAG-TCC-3'	SEQ ID 12
Secondary sequencing primers		
Seq1F	5'-CAG-ACC-AGA-GCC-AAC-AGC-CCC-3'	SEQ ID 13
Seq2A	5'-CAC-TCT-TTG-GCA-ACG-ACC-C-3'	SEQ ID 14
Seq3A	5'-GGT-ACA-GTA-TTA-GTA-GGA-CC-3'	SEQ ID 15
Seq5A	5'-GTA-CTG-GAT-GTG-GGT-GAT-GC-3'	SEQ ID 16
Seq7A	5'-GTG-GGA-AAA-TTG-AAT-TGG-G-3'	SEQ ID 17
PCR2.3	5'-CTA-ACT-GGT-ACC-ATA-ATT-TCA-CTA-AGG-GAG-G-3'	SEQ ID 4
Seq2B	5'-GGG-TCA-TAA-TAC-ACT-CCA-TG-3'	SEQ ID 18
Seq4B	5'-GGA-ATA-TTG-CTG-GTG-ATC-C-3'	SEQ ID 19
Seq6B	5'-CAT-TGT-TTA-ACT-TTT-GGG-CC-3'	SEQ ID 20
Seq7B	5'-GAT-AAA-ACC-TCC-AAT-TCC-3'	SEQ ID 21
Seq4A	5'-GTA-CAG-AAA-TGG-AAA-AGG-3'	SEQ ID 22
Seq6A	5'-GGA-TGA-TTT-GTA-TGT-AGG-3'	SEQ ID 23
Seq5B	5'-GGA-TGT-GGT-ATT-CCT-AAT-TG-3'	SEQ ID 24

Table 2 : Replacement or secondary sequencing primers. Initial preferred sequencing primers can be replaced by a set of possible replacement primers. Suggestions are indicated in the table.

Initial sequencing primer	Preference set of replacement sequencing primers
Seq1FOR	Seq1F & Seq2A
Seq2FOR	Seq3A & Seq5A
Seq3F	Seq5A & Seq7A

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Initial sequencing primer	Preference set of replacement sequencing primers
Seq1B	PCR2.3 & Seq2B
Seq3B	Seq2B & Seq4B
Seq6R	Seq6B & Seq7B

Table 3. Overview of mutations present in a clone used for training and validation of the assay.

PROTEASE	REVERSE TRANSCRIPTASE
V003I	V035M
L010I	M041L
I013V	K103N
K020R	E122K
E035D	I135T
M036I	M184V
S037N	G196E
K043T	L210W
F053L	R211K
I054V	L214F
L063P	T215Y
I064V	P225H
I066F	K238T/K
A071V	P272A
V082T	T286A
I084V	V292I
	I293V
	F346Y
	M357T
	R358K
	K366R
	T376S

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Mutations were revealed according to the method of the present invention. The numbering corresponds to the exact amino acid location in either the protease or reverse transcriptase. The amino acids are represented by their one letter code. This

code is well known in the art (see Alberts et.al The Molecular Biology of the Cell, 1994)

5 Table 4. On overview of patient samples comprising several mutations present in the protease and reverse transcriptase domain of HIV.

Mutations were revealed according to the method of the present invention. The numbering corresponds to the exact amino acid location in either the protease or reverse transcriptase.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
PROTEASE	V003I	V003I	V003I	V003I	V003I	V003I	V003I	V003I	V003I	V003I
	L010I	L010I	L010I	L010I	L010I	L010I	L010I	L010F/I	L010F	L010I
	L024I	T012K	I015V	I013V	I015V	I015V	T012A/T	I015V	I013V/I	I013V
	S037N	L019V	K020R	K020I	K020T	K020V	I013V	L019I	K020R	L033F
	G048M	K020R	E035D	S037N	L024F	E035D	L019I	K020I	V032I	E035D
	F053L	E034Q	M036I	P039Q	S037N	S037D/N	K020R	M036I	E035D	M036I
	I062V	E035D	S037K	R041K	M046I	R041R/K	E035D	S037N	M036I	S037N
	L063P	M036I	R041N	M046I	I054L	M046L	M036I	M046I	S037D/N	R041K
	I064V	S037N	M046I	I054V	I062V	G048V	S037N	I054V	R041K	K043T/K
	E065D	R041K	L063P	I062V	L063P	F053Y	R041K	D060E	K043T	I054V
	I072V	G048V	H069K	L063P	A071V	I054V	K045R/K	I062V	M046M/I	D060E
	T074S	I054S	A071V	H069R	I072L	K055R/K	I062V	L063P	I054V	I062V
	V077I	I062V	T074S	A071L	G073S	Q061H	L063P	A071V	K055R/K	L063P
	V082A	L063P	V082F	I072V	V077I	L063D	H069H/Q	V082T	D060E	A071V
		A071I	N088E	T074P	I084V	A071T	L089M	I084V	L063P	I072L
		I072T/I	L089M	V077I	I085V/I	I072V	L090M	I085V	I064V	G073S
		T074S	L090M	I084V	L089V	V077I	I093L/I	L090M	I072V/I	P079P/S
		V082A	I093L	L090M	L090M	V082A			I084V	L090M
		L090M		I093L	I093L	I085V			I085V/I	
		I093L				L090M	Q092K		L090M	
REVERSE	P004S	I002V/I	P001P/L	K020R	M041L	M041L	V035T	K011T/K	D017D/E	P004S
	K011R	V035M	K020R	A033G	K043N	K043E	M041L	K020R/K	M041L	V035I
	V021I	T039A	V035T	V035L/M	E044D	E044A	K043N/K	V035A/V	K043Q	M041L
	T039A	E040F	T039R	T039A	A062V	D067N	T069D/N	T039A	E044D	E044D

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
	M041L	M041L	M041L	M041L	D067N	T069D	K070R/K	M041L	K046R/K	D067N
	K043E	K043E	K043E	D067S/N	K070R	K102Q	E122K	E044D	V060I	T069D
	E044A	D067N	E044D	K070R	L074I	V108V/I	D123E	D067N	D067G	K070R
	I050T	T069D	V060I	L074I	V075T	V118I	I135V	T069D	T069D	V106I
	D067D/N	V118I	D067N	V075M	K101E	I135T	K173A	V075V/M/I	L074V	F116L/F
	L074I	I135T	T069D	A098G	V108I	S162C/S	Q174K	K082R/K	K103N	S162C
	V075M	S162Y	A098G	K101E	V118I	I178M/I	D177E	A098G	V111I	D177E
	K101Q	V179I	V118I	D123S	S162Y	V179I	Y181C	K103N	V118I	I178L
	V108I	Y181C	D121H	I167V	Y181C	Y181C	G190A	V118I	D123D/N	V179V/I
	V118I	M184V	I135T/I	D177E	M184V	I202V	Q207E	D121H	I135T	Y181C
	D123D/E	T200E	I142V	V179I	V189V/I	H208F	L214L/F	D123S	V179A/V	Y188L
	I142V	E203K	D177E	M184V	G190A	L210W	T215F/C	I142V	Y181C	T200A
	S162C	Q207E	V179V/I	G190A	Q197E	R211K	L228R/H	S162C	M184V/M	Q207K
	D177E	L210S	Y181C	I195L/I	H208Y	L214F	V245Q	D177E	G196E	L210W
	V179I	R211K	G190G/A	G196E	L210W	T215Y	E248D	I178L	E203K	R211A
	Y181C	L214F	T200A	I202V	R211K	H221Y	D250S	M184V	Q207E	L214F
	M184V	T215Y	E203D	H208Y	L214F	L228H	K275R	T200A	L210W	T215Y
	G190A	D218E	Q207E	L214F	T215Y	I257L	R277K	E203D	R211K	D218E
	L193M	K219Q	H208F/Y	T215F	D218E	P272A	Q278H	H208Y	L214F	K219E
	G196E	L228H	L210W	K219Q	K219Q	T286A/T	K281R	L210W	T215Y	P272A
	T200A	V245M	R211K	L228H	L228H	A288S	T286A/T	R211K	K219R	Q278E
	E203K	P272A	L214F	V245E	Q242H	I293V	E291D	L214F	K223Q	E291D
	H208Y	K275Q	T215Y	R277K	P272A	Q334E	I293V	T215Y	P243T	V292I
	L210W	V276T	L228H	T286P/T	R277K	G335S	E297A	V245E	V245K	I293V
	R211K	L283I	V245Q	E297K	E297Q	R356K	G335D	R277K	D250E	Y318F
	L214F	I293V	S251S/T	D324E	D324E	M357R	E344D/E	T286A	R277K	Q334L
	T215Y	E297R/K	E291D	K347R/K	I341F	R358K	F346H/Y	I293V	I293V	P345Q
	K219N	D324E	I293V	A355T	N348I	Q367E	R356K	Y318F	M357T	N348I
	K223E	I329L	P294T	M357V	A360T	I375V	M357R	D324E	R358K	K350R
	F227L	R356K	G335D	G359S	D364E	T376A	G359S/T	G335D	T376C	V365I
	L228R	M357L	R356K	V365V/M/I	T376A	T386I	T362S/T	M357K	T377N	E370A
	V245T	A360T	G359T	E370D	T377K	K390R	K366R	I375V	K390R	T376A
	R277R/K	T376A	T376A	T376A	K390R	T400A	A371V	T376A	T400A	I380V/I
	T286A/P	E399G	T377Q	S379C	E399D		T376A	T386I		T386I
	P294Q		K390R	V381V/I	T400A		T386I	K390R		K390R
	E297K		T400A	K390R			K388T	E399D		

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
	D324D/E G359S T369A T376S K390R						K390R			

Examples

The examples given below are to exemplify the present invention and not to limit the scope of the invention.

5

Modes for carrying out the invention:

I. Introduction

The choice of oligonucleotides or primers for amplification and sequencing of the target nucleic acid is critical for the sensitivity and and specificity of the assay. The sequence to be amplified is usually only present in minute amounts in a complex matrix e.g. a blood sample of a patient. The primers should therefor be sufficiently complementary to the target sequence to allow efficient amplification and subsequent sequencing of the target nucleic acid. If the primers do not anneal properly to the target area, the amplification can be seriously affected, and as a consequence yield false results. It has been found that the reliability of primer dependent analyses can be further enhanced if the primer sequences meet or are optimized with respect to the following requirements: G and C content, no duplex formation between the primers, no hairpin formation within the primer(s), no false priming sites for the selected set of nucleotides, no hybridization with itself. It is evident that the requirements also concern the complementary strands. Evidence has been reported that sequencing primer do not need to match exactly the target sequence (Kwok et al. 1990).

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A. Amplification of the HIV-1 Protease -Reverse transcriptase coding domain

RNA was isolated from 100 μ l of plasma according to the method described by Boom et al. (1990), and reverse transcribed with the GeneAmp reverse transcriptase kit (Perkin Elmer) as described by the manufacturer using a HIV-1 specific downstream primer (OUT3, see Table 1). Two subsequent nested PCR were set up using specific outer primers (PRTO-5 and OUT3) and inner primers (PCR2.5 and PCR2.3), respectively (see Table 1). The outer primer reaction was done as described in WO97/27480 and Hertogs et al. Antimicrob. Agents Chemotherap. 1998. The inner amplification was performed in a 96 well plate as follows: 4 μ l of the outer amplification product was diluted to a final volume of 50 μ l using a 10X amplification

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mix consisting of 5 μ l 10X PCR buffer containing 15 mM MgCl₂, 1 μ l dNTP's (10 mM) 0.5 μ l PCR2.5 (0.25 μ g/ml), 0.5 μ l PCR2.3 (0.25 μ g/ml), 0.4 μ l Expand High Fidelity (3.5 U/ μ l) and MQ water. Amplification was initiated after a short denaturation of the amplification product made using the outer primers (2 min at 94°C).
5 10 amplification cycles were started consisting of a 15 sec denaturation step at 94°C, a 30 sec annealing step at 60°C and a 2 min polymerase step at 72°C, respectively. This amplification was immediately followed by 25 cycles consisting of a 15 sec denaturation step at 94°C, a 30 sec annealing step at 60°C and a x min polymerase step at 72°C, respectively; where x started at 2 min and 5 sec and increased each cycle with
10 5 sec. Amplification was finalised by an additional polymerase step (7 min at 72°C). Subsequently, the reaction was held at 4°C till further analysed or stored at -20°C (for short periods) or -70°C (for longer periods). In order to analyse the amplification products, a DNA agarose gel was run and amplification products were visualised using UV-detection. Obtained PCR products were purified using the QIAquick 96-well plate
15 system as described by the manufacturer (Qiagen).

B. Sequencing of pol coding region

The coding domain of the pol gene present on the amplified fragments was analysed via sequencing using standard sequencing techniques. Preferentially, one
20 started initial with a set of 6 primers (Seq1FOR, Seq2FOR, Seq3F, Seq1B, Seq3B and Seq6R) covering the coding domain of the HIV-protease and reverse transcriptase protein. Sequences and location onto the coding region are shown in Table 1 and Figure 1, respectively. The sequencing was started by first distributing 4 μ l of the primer stocks (4.0 μ M) over a 96 well plate where each stock is pipetted down the
25 column. In a second step, master mixes were made consisting of 14 μ l MQ, 17.5 μ l dilution buffer, 7 μ l sample (PCR fragment) and 14 μ l Big Dye Terminator Mix. A fraction (7.5 μ l) of each master mix, containing a specific PCR fragment, was transferred to a specific place into the 96 well plate so that each sample fraction was mixed with a different PCR primer set. Samples were pipetted across the rows.
30 Samples were placed in a thermal cycler and sequencing cycles started. The sequencing reaction consisted of 25 repetitive cycles of 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C, respectively. Finally, sequence reactions were held at 4°C till further analysis or stored as previously described. The sequencing reactions were precipitated using a standard ethanol precipitation procedure, resuspended in 2 μ l
35 formamide and heated for 2 minutes at 92°C in the thermal cycler. Samples were cooled on ice until ready to load. 1 μ l of each reaction was loaded on a 4.25% vertical acrylamide gel in a 377 sequencer system and gel was run until separation of the fragments was complete.

C. Sequence analysis of pol coding region

Sample sequences were imported as a specific project into the sequence manager of Sequencher (Genecodes) and compared to the wild type HXB2 Pro/RT reference sequence (*e.g.* HIVHXB2 sequence, Genbank sequence accession No. 327742). Sequences were assembled automatically and set at 85% minimum match. Secondary peaks were searched and the minimum was set at 60%. Any sequence that hung over the 5' end or the 3' end of the reference was deleted. When a region of overlap between sequences from the same strand was reached, the poorest quality of sequence was deleted leaving an overlap of 5-10 bases. Ambiguous base calls are considered poor matches to exact base calls. The sequence assembly was saved within a contig that can be edited.

Obtained sequences were edited so that base calls could be interpreted easily. Ambiguous sequences were retrieved and checked for possible errors or points of heterogeneity. When the point of ambiguity appeared correct (both strands of sequence agree but is different from the reference sequence) it was interpreted to be a variant. The reference sequence was used as an aid for building a contig and a guide to overall size and for trimming, but was not used for deciding base calls. A change was only made when both strands agreed. All gaps were deleted or filled, unless they occur in contiguous groups of a multiple of 3 (I.E. insertion or deletion of complete codons) based on data from both sequence strands. Once the editing was complete, the new contig sequence was saved as a consensus sequence and used for further analysis.

Detailed sequence editing was performed following certain rules: A) ABI primer blobs are trimmed at 5' ends where 1 consecutive base remain off the scale; sequence is trimmed not more than 25% until the first 25 bases contain less than 1 ambiguity; at least first 10 bases from the 5' end are removed, B) 3' ends are trimmed starting 300 bases after the 5' trim; the first 25 bases containing more than 2 ambiguities are removed; trim from 3' end until the last 25 bases contain less than 1 ambiguity. The maximum length of the obtained sequence fragment after trimming is 550 bases.

Sequences that failed to align were removed from the assembly and replaced by data retrieved from new sequence analyses. When further failures occurred, PCR reactions were repeated. Chromatograms were visualized using the IBM software system (cfr. Table 3 & 4).

D. Detection of clonal clinical samples- analysis of limit of detection for heterozygous base calls.

A clonal clinical sample was mixed with wild type HXB2 at known ratio's to

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determine limits of detection of the system. The limit of detection was found to be around 1000 RNA copies/ml from plasma; mixed populations of mutations could be detected when present down to 20%.

5 The references cited in the specification are listed below :

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SEQUENCE LISTING

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<120> METHOD FOR MUTATION DETECTION IN HIV-1 USING POL
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What is claimed is:

1. A method for mutation analysis of the pol gene of HIV-1 isolates comprising the steps of:
 - 5 a) isolation of a sample,
 - b) virion RNA extraction of the isolated sample material
 - c) amplifying RNA via nested PCR using outer primers as represented in SEQ ID No.1 and 2 to obtain a primary PCR product,
 - 10 d) amplifying said primary PCR product via nested PCR using a 5' and 3' primer chosen from the inner primers as represented in SEQ ID No. 3 , 4, 5 and 6, and
 - e) sequencing this secondary obtained PCR product using at least one sequencing primer chosen from any of SEQ ID No. 7 to 12.
- 15 2. A method for mutation analysis of the pol gene of HIV-1 isolates comprising the steps of:
 - a) isolation of a sample,
 - b) viral DNA extraction of the isolated material
 - c) amplifying DNA via nested PCR using outer primers as represented in SEQ ID No.1 and 2 to obtain a primary PCR product,
 - 20 d) amplifying said primary PCR product via nested PCR using a 5' and 3' primer chosen from the inner primers as represented in SEQ ID No. 3 , 4, 5 and 6, and
 - e) sequencing this secondary obtained PCR product using at least one sequencing primer chosen from any of SEQ ID No. 7 to 12.
- 25 3. Method according to Claim 1 or 2, wherein one of the initial sequencing primers is replaced by one or a pair of replacement primers.
- 30 4. Method according to any of claims 1 to 3 wherein the sequencing primer is chosen up to 1, 2, 3 or 4 nucleotides upstream or downstream the described primer region
5. Method according to any of claims 1 to 3 wherein the outer primer is chosen up to 1, 2, 3 or 4 nucleotides upstream or downstream the described primer region.
- 35 6. Method according to any of claims 1 to 3 wherein the inner primer is chosen up to 1, 2, 3 or ' nucleotides upstream or downstream the described primer region.

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7. Method according to any of claims 1 to 6 wherein the sample contains free virion particles or virus infected cells.
- 5 8. A primer as represented in SEQ ID. No. 1 and 5 to 12 used to analyse the sequence of the HIV pol gene of HIV-1 isolates
9. A primer as represented in SEQ ID No. 13 to 24 to analyse the sequence of the HIV pol gene sequence of HIV-1 isolates
- 10 10. A primer according to claim 8 or 9 comprising the complementary strands.
11. An oligonucleotide used as primer having at least 80% sequence similarity to the sequences represented in SEQ ID 1-24
- 15 12. An oligonucleotide used as primer having at least 90% sequence similarity to the sequences represented in SEQ ID No. 1-24
13. An oligonucleotide used as primer having at least 95% sequence similarity to the sequences represented in SEQ ID No. 1-24.
- 20 14. A primer according to claim 9 for use in the method of claim 1 or 2.
15. An oligonucleotide used as primer comprising at least 8 consecutive nucleotides, wherein said at least 8 consecutive nucleotides are present in SEQ ID No. 1-24
- 25 16. An oligonucleotide according to claim 10 wherein said sequence is present in SEQ ID No. 5-24.
17. Method according to any of claims 1 to 6 wherein the sequencing is performed on the primary PCR product.
- 30 18. Method according to any of claims 1 to 6 wherein the mutation identified confers resistance to an antiretroviral
- 35 19. Method according to any of claims 1 to 6 wherein the mutation identified confers resistance to a protease inhibitor inhibitor
20. Method according to any of claims 1 to 6 wherein the mutation identified confers resistance to a reverse transcriptase inhibitor
- 40

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21. Method according to any of claim 1 to 6 wherein the mutation identified confers resistance to an integrase inhibitor.
- 5 22. Diagnostic kit for the mutation analysis of the HIV pol gene of HIV-1 isolates comprising at least one of the primers having the sequence as represented in SEQ ID No. 1 to 12.
23. Diagnostic kit for the mutation analysis of the HIV pol gene of HIV isolates comprising at least one primer selected from SEQ ID No. 13 to 24.

10

Virco Sequencing Strategy

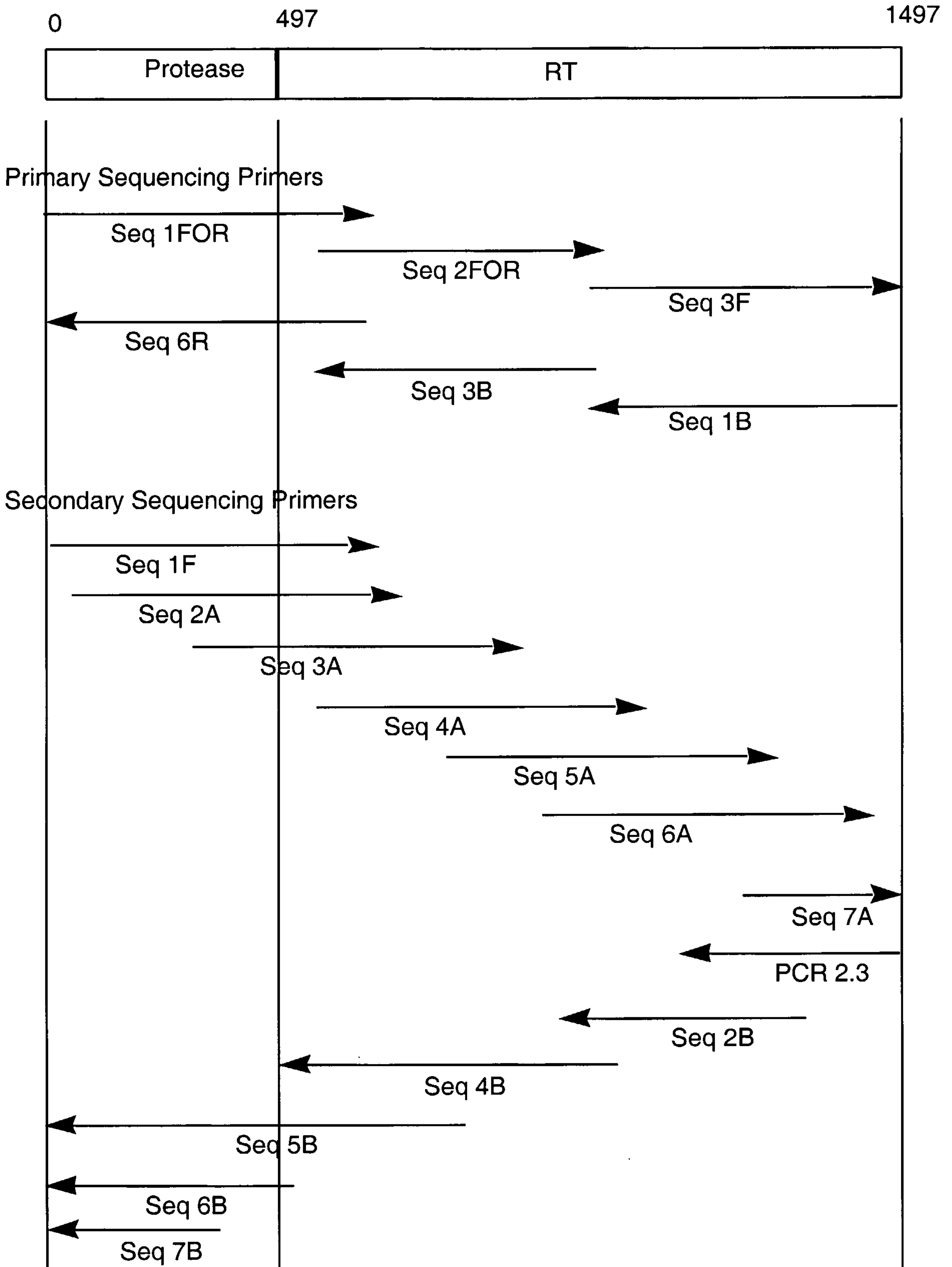


Figure 1