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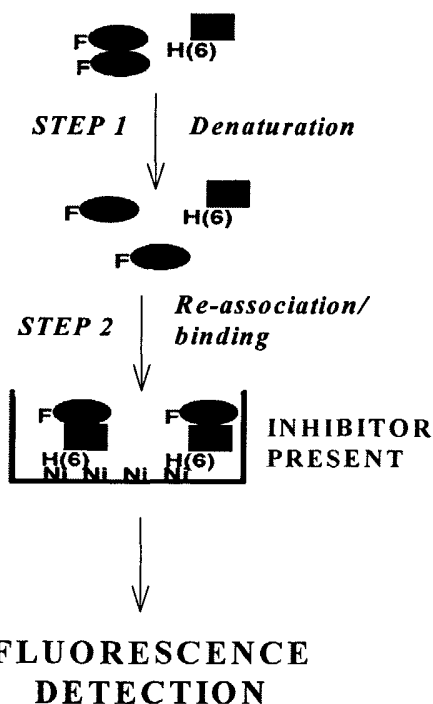


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(57) Abstract: A method for measuring heterodimerization of HIV RT, which comprises the steps of: a) providing a first solution comprising p66 subunit homodimers in the presence of a dissociation agent; b) contacting the first solution with p51 RT subunits and incubating in the presence of a reassociation buffer to allow association of a complex of p66/p51 RT subunits, wherein one of the subunits comprises an affinity tag and the other of the subunits comprises a detectable label; c) contacting the incubate of step b) with an affinity medium under conditions that enable the p66/p51 complex to bind to the affinity medium; and d) determining the amount of complex formed by measuring the level of detectable label bound to the affinity medium (or by measuring the reconstituted RT polymerase activity). Steps a) to d) can be carried out in the presence or absence of a test compound followed by e) comparing the test compound sample to a control sample lacking the compound, whereby modulated p66/p51 complex formation in the test compound sample is indicative of the ability of the compound to modulate, inhibit or enhance heterodimerization. The method can be used to screen for inhibitors of HIV RT dimerization.

ASSAY FOR IDENTIFYING INHIBITORS OF HIV RT DIMERIZATION

FIELD OF THE INVENTION

- 5 The present invention relates to a new assay to measure the process of HIV-1 reverse transcriptase (RT) dimerization. This invention particularly relates to a new method suitable for adaptation to high-throughput screening for inhibitors of this process.

10 BACKGROUND OF THE INVENTION

- Reverse transcriptase (RT) of the human immunodeficiency virus type 1 (HIV-1) plays a key role in the replication of HIV by converting single-stranded genomic RNA into double-stranded proviral DNA and represents one of the main targets for the development of AIDS therapy. Most inhibitors of RT described in the past years,
15 whether nucleoside analogues or non-nucleoside inhibitors target the polymerase activity of RT but present some limitations including toxicity and the emergence of resistant strains.

- The biologically relevant and active form of HIV RT found in infectious virions is a
20 heterodimer containing two polypeptides, p66 and p51; the latter derived from the former by proteolytic cleavage of its C-terminal domain during viral maturation. The two subunits of 66 and 51 kDa form are present in a 1 to 1 ratio. This heterodimeric RT is produced in a two-step dimerization process, the kinetics of which involve the rapid association of the two subunits into an immature dimer, followed by a slow
25 conformational change yielding the fully active form ($p66 + p51 \rightarrow p66/p51$ immature $\rightarrow p66/p51$ active; Divita et al., **1995**, J. Mol. Biol. 245, 508-52112, 13).

- The DNA polymerase and RNase H activities of HIV-1 RT are dependent on the dimeric structure of the enzyme (Restle et al., **1990**, J. Biol. Chem. 265, 8986-8988;
30 Restle et al., **1992**, FEBS Letters 300, 97-100). Because dimerization of these subunits is required for enzymatic activity, interference with the dimerization of HIV-1 RT could constitute an appropriate target for the development of anti-HIV compounds. A case in point: synthetic peptides from the thumb domain of the p51 subunit of RT inhibit the "maturation" process (Morris et al., **1999**, Biochemistry 38,
35 15097-15103). Compounds that interfere with the formation and/or stability of the RT

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dimer may therefore represent a novel class of antiviral compounds.

Several publications disclose association-dissociation assays for measuring the kinetics of the p51-p66 dimerization process (Cabodevilla et al., **2001**, Eur. J. Biochem. 268:1163-1172; Morris et al., **1999**, J. Biol. Chem. 274(35), 24941-24946; Divita et al., **1995**, Biochemistry 34, 16337-16346; Divita et al., **1994**, J. Biol. Chem. 269(18), 13080-13083; Divita et al., **1993**, FEBS Letters 324(2), 153-158; Becerra et al., **1991**, Biochemistry 30, 11707-11719). They all proceed to measure the dimerization by either: size-exclusion HPLC; measuring the RNA-dependent DNA polymerase activity of the sample; immunoprecipitation; or by monitoring intrinsic fluorescence emission of the protein. None of these publications disclose a binding assay suitable for HTS format.

Tachedjian et al., **2000** (PNAS 97(12) 6334-6339) disclose a yeast 2-hybrid system to study the association-dissociation process of RT dimerization. However, this system is not easily amenable to a high throughput assay for assessing large numbers of potential inhibitors.

Howard et al., **1991**, J. Biol. Chem. 266(34), 23003-23009 suggest an assay system to monitor therapeutic agents that act by preventing dimer formation. Again, this publication does not disclose a binding assay amenable for high throughput screening suitable for the identification of potential inhibitors of heterodimerization.

SUMMARY OF THE INVENTION

The present invention provides a high-throughput assay suitable for assessing large numbers of compounds for their activity against the dimerization of HIV RT.

- 5 The general principle of the assay of the present invention involves dissociating a p66/p66 RT homodimer (appropriately labeled with a detectable moiety and/or affinity tagged) in the presence of limiting concentrations of a dissociation agent (i.e. a denaturant such as urea), contacting it with the p51 RT subunit and incubating the mixture in the presence of an excess denaturant-free (or denaturant reduced) buffer
- 10 to allow re-association of the RT subunits, with subsequent affinity capture of any reconstituted p51/p66 RT heterodimer. After washing to remove unbound material, the amount of affinity-associated material is assessed by measuring the level of a detectable moiety (or alternatively, by measuring the reconstituted RT polymerase activity in which case the p66 subunit does not necessarily require labeling), and is
- 15 proportional to the amount of labeled p66/p51 RT heterodimer bound by affinity. This assay is performed in the presence or absence of a test compound whereby a modulation (decrease/increase) in binding of p66 monomer subunit to p51 subunit in the presence of the test compound compared to the control is indicative that the test compound is a modulator of RT dimerization.

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In a first aspect of the present invention, there is provided a method for measuring heterodimerization of HIV RT, which comprises the steps of:

- a) providing a first solution comprising p66 subunit homodimers in the presence of a dissociation agent;
- 25 b) contacting said first solution with p51 RT subunits and incubating in the presence of a reassociation buffer to allow association of a complex of p66/p51 RT subunits, wherein one of said subunits comprises an affinity tag and the other of said subunits comprises a detectable label;
- c) contacting the incubate of step b) with an affinity medium under conditions
- 30 that enable the p66/p51 complex to bind to said affinity medium; and
- d) determining the amount of complex formed by measuring the level of detectable label bound to the affinity medium (or by measuring the reconstituted RT polymerase activity).

- 35 In a second aspect of the present invention, there is provided a method for

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identifying compounds capable of modulating the HIV RT heterodimerization, comprising:

- carrying out steps a) to d) described above in the presence or absence of a test compound; and
- 5 e) comparing the test compound sample to a control sample lacking said compound, whereby modulated p66/p51 complex formation in the test compound sample is indicative of the ability of said compound to modulate heterodimerization.

- 10 In a third aspect of the present invention, there is provided a method for identifying compounds capable of interfering with the HIV RT heterodimerization, comprising:
- carrying out steps a) to d) described above in the presence or absence of a test compound; and
- 15 e) comparing the test compound sample to a control sample lacking said compound, whereby decreased p66/p51 complex formation in the test compound sample is indicative of the ability of said compound to inhibit heterodimerization.

- In a fourth aspect of the present invention, there is provided a method for identifying
- 20 compounds capable of enhancing the HIV RT heterodimerization, comprising:
- carrying out steps a) to d) described above in the presence or absence of a test compound; and
- e) comparing the test compound sample to a control sample lacking said compound, whereby increased p66/p51 complex formation in the test
- 25 compound sample is indicative of the ability of said compound to enhance heterodimerization.

According to a fifth aspect of the present invention, there is provided a method for measuring homodimerization of HIV RT, which comprises the steps of:

- 30 a) providing a first solution comprising first p66 subunit homodimers in the presence of a dissociation agent;
- b) contacting said first solution with second p66 subunits homodimers, in the presence of said dissociation agent, and incubating in the presence of a reassociation buffer to allow association of a complex of p66/p66 RT
- 35 subunits, wherein one of said subunits comprises an affinity tag and the other

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of said subunits comprises a detectable label;

c) contacting the incubate of step b) with an affinity medium under conditions that enable the p66/p66 complex to bind to said affinity medium; and

5 d) determining the amount of complex formed by measuring the level of detectable label bound to the affinity medium (or by measuring the reconstituted RT polymerase activity).

In a sixth aspect of the present invention, there is provided a method for identifying compounds capable of modulating the HIV RT homodimerization, comprising:

10 carrying out steps a) to d) described above in the presence or absence of a test compound; and

e) comparing the test compound sample to a control sample lacking said compound, whereby modulated p66/p66 complex formation in the test compound sample is indicative of the ability of said compound to modulate
15 homodimerization.

In a seventh aspect of the present invention, there is provided a method for identifying compounds capable of interfering with the HIV RT homodimerization, comprising:

20 carrying out steps a) to d) described above in the presence or absence of a test compound; and

e) comparing the test compound sample to a control sample lacking said compound, whereby decreased p66/p66 complex formation in the test compound sample is indicative of the ability of said compound to inhibit
25 homodimerization.

In a eighth aspect of the present invention, there is provided a method for identifying compounds capable of enhancing the HIV RT homodimerization, comprising:

30 carrying out steps a) to d) described above in the presence or absence of a test compound; and

e) comparing the test compound sample to a control sample lacking said compound, whereby increased p66/p66 complex formation in the test compound sample is indicative of the ability of said compound to enhance
35 homodimerization.

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According to a ninth aspect of the present invention, there is provided a kit for testing compounds potentially modulating the HIV RT heterodimerization, said kit comprising a plurality of affinity-tagged p66 subunit homodimers, a plurality of labeled-p51 RT subunits, a dissociation agent, a reassociation buffer, an affinity medium and instructions on how to use said subunits for identifying test compounds binding to said transcriptase.

According to a tenth aspect of the present invention, there is provided a kit for testing compounds potentially modulating the HIV RT homodimerization, said kit comprising a plurality of affinity-tagged p66 subunit homodimers, a plurality of labeled-p66 RT subunits, a dissociation agent, a reassociation buffer, an affinity medium and instructions on how to use said subunits for identifying test compounds binding to said transcriptase.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of the preferred embodiments with reference to the accompanying drawings which is exemplary and should not be interpreted as limiting the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and in which:

Figure 1 illustrates the effect of urea concentration on HIV-1 p66/p66 RT protein structure. Panel A illustrates the change in intrinsic protein fluorescence emission maximum wavelength as a function of urea concentration. The increase in the F350/335 ratio indicates an increased exposure of normally 'buried' tryptophan residues to aqueous solvent. Panel B illustrates results of circular dichroism analyses. The increase in molar ellipticity at 222 nm is a measure of the loss of RT secondary structure. Panel C shows the change in 8-anilino-1-naphthalene-sulfonic acid (ANS) binding to RT, which correlates with the subunit integrity of the RT dimer.

Figure 2 illustrates the dimerization state of p66 subunit as a function of urea concentration as determined by size-exclusion chromatography.

Figure 3 illustrates the optimization of urea concentration for use in the TRF (Time Resolved Fluorescence) assay for HIV-1 RT p66/p51 heterodimer formation.

- 5 **Figure 4** illustrates the specific TRF signal as a function of time following dilution of urea-denatured His6x-p51 RT subunit and Eu-N1-iodoacetamido chelate labeled p66 RT subunit into "subunit association" buffer containing 100 mM MgCl₂. Details are described in the examples.
- 10 **Figure 5** illustrates the assay concept (Panel A) and Inhibition of HIV-1 RT dimerization by TSAO-m³T and other NNRTIs (Panel B), as measured by TRF.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

- 15 Unless otherwise defined, all technical and scientific terms used herein have the same meaning as those commonly understood by one of ordinary skill in the art to which the invention pertains.

As used herein, the terms "label", "detectable label" or "detectable marker" refer to
20 any group that may be linked to the p66 or p51 to allow recognition either directly or indirectly of the subunit such that it can be detected, measured and quantified. Examples of such "labels" include, but are not limited to, fluorescent labels, chemiluminescent labels, colorimetric labels, enzymatic markers, radioactive isotopes and affinity tags such as biotin. Such labels are attached to the p66 or p51
25 by well known methods. A label, or multiple labels, of the present invention can be introduced at any position on the p66 or the p51, for example, the label can be at either the C- or N-termini or within the main chain of the protein.

The term "affinity label" or "affinity tag" as used herein refers to a label, which is
30 specifically trapped by a complementary affinity ligand. Examples of pairs of affinity tag/affinity ligand include but are not limited to: Maltose-Binding Protein (MBP)/maltose; Glutathione S Transferase (GST)/ glutathione; streptavidin tag/streptavidin or neutravidin, or histidine (His)/ metal. The metal used as affinity ligand may be selected from the group consisting of: cobalt, zinc, copper, iron, and nickel
35 (Wong et al. (1991) Separation and Purification Methods, 20(1), 49-106). The affinity

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tag of the present invention can be introduced at any position on the p66 or the p51, for example, the tag can be at either the C- or N-termini or within the main chain of the protein, but preferably on the N-terminus of the protein. Preferably, the metal selected is nickel. The affinity ligand can be set up in solid phase such as: beads,
5 microplate wells or columns to facilitate separation by affinity chromatography. Such affinity tags may be introduced recombinantly to p66 or p51 by well-known methods.

The terms "monomer" and "monomeric" p66 or "monomeric" subunit refer to a single subunit of a dimeric p66. The monomeric subunit may be an exact copy of the
10 naturally occurring monomeric subunit or it may be either a biologically active analog or a biologically inactive analog (negative dominant).

As used herein, the term "subunit", when referring to either p66 or p51, is intended to mean one or two constituents of homodimeric RT (p66/p66) or heterodimeric RT
15 (p66/p51).

As used herein, the term "homodimer" refers to a dimeric molecule wherein both subunits are the same, for example p66/p66.

20 As used herein, the term "homodimerization" refers to the process by which same subunits, namely p66, dimerize.

As used herein, the term "heterodimer" refers to a dimeric molecule wherein the two subunit constituents are different, for example p66 and p51.
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As used herein, the term "heterodimerization" refers to the process by which two different subunits, namely p66 and p51, dimerize.

As used herein, the term "dissociation agent" refers to a substance that is capable of
30 dissociating a multicomponent complex into its individual subunits.

As used herein, the term "reassociation buffer" refers to a buffer that favors association of the individual subunits into a multicomponent complex.

35 As used herein, the term "denaturant free" refers to conditions in which the

dissociation agent is no longer present in quantities sufficient to prevent association of the majority of subunits.

As used herein, the term "affinity medium" is intended to mean a solid support such as microplate wells, beads, or column which are coated with an affinity ligand that is suitable to capture an affinity tag.

PREFERRED EMBODIMENTS

In a first preferred aspect of the first embodiment, there is provided a method for measuring heterodimerization of HIV RT, wherein preferably, the first solution in step a) comprises a buffer selected from Tris-HCl, HEPES or bis-Tris. More preferably, the buffer is Tris-HCl at a concentration of between 0mM and 50mM. Most preferably, the buffer is Tris-HCl at a concentration of 25mM.

Preferably, the first solution in step a) further comprises a salt selected from Na_2SO_4 or MgCl_2 . More preferably, the salt is MgCl_2 at a concentration of between 0mM and 100mM.

In a preferred aspect of the first embodiment in step a), the dissociation agent is selected from urea, guanidinium-HCl or acetonitrile. More preferably, the dissociation agent is urea at a concentration from 1 to 3.5 M.

Preferably, the re-association buffer in step b) is the same buffer used to dissociate the p66/p66 subunits but is preferably denaturant-free. More preferably, the re-association buffer is present in excess amount to the denaturant buffer to dilute the dissociation agent sufficiently to allow re-association of p66 and p51 subunits. Most preferably, the re-association buffer is added in a 10-fold excess over the dissociation buffer.

Preferably, the re-association buffer in step b) further comprises a salt selected from Na_2SO_4 or MgCl_2 . More preferably, the salt is Na_2SO_4 at a concentration of between 0mM and 100mM. Most preferably, the salt is Na_2SO_4 at a concentration of 50mM.

Preferably, the p51 in step b) is affinity-tagged or has a detectable label. More preferably, the p51 in step b) is affinity-tagged.

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Preferably, the p66 in step b) has a detectable label or is affinity-tagged. More preferably, the p66 in step b) has a detectable label.

- 5 Preferably, the affinity-tag in step b) is selected from the group consisting of: a hexa-histidine tag, FLAG, T7, HA, GST, or biotin. More preferably, the affinity tag is hexa-histidine.

- 10 In another preferred aspect if the first embodiment in step b), the detectable label is selected from the group consisting of: a fluorescent label (such as fluorescein, Oregon green, rhodamine, Texas-red, phycoerythrin or Eu^{3+}), a radioactive atom (such as ^3H or ^{125}I), a chemiluminescent label (such as luciferase), a detectable antibody specific to p66 or specific to a tag attached to p66 (preferably, the tag on p66 is selected from the group consisting of: a hexa-histidine tag, FLAG, T7, HA,
- 15 GST, or biotin), and an enzymatic marker (such as β -galactosidase or horseradish peroxidase). More preferably, the label is a fluorescent label selected from the group consisting of: fluorescein, Oregon green, rhodamine, Texas-red, phycoerythrin and Eu^{3+}). Most preferably, the fluorescent label is Eu^{3+} .
- 20 Preferably, the detectable label in step b) is added to a cysteine residue that is introduced recombinantly in place of a proline residue at the N-terminus of p66.

- The detectable label is measured by appropriate means such as fluorometer, radioactivity counter, colorimeter or chemiluminometer as will be readily recognized
- 25 by a person of skill in the art.

- In another preferred aspect of the first embodiment in step c), the affinity medium is a solid phase such as microplate wells or beads coated with an affinity-ligand that is suitable to capture the affinity tag. More preferably, the microplate wells are coated
- 30 with a receptor selected from the group consisting of: Ni^{2+} , anti-tag antibodies, glutathione or streptavidin. Most preferably, the microplate wells are coated with Ni^{2+} , and preferably the affinity tag is a histidine tag. A person skilled in the art will recognize the suitable combinations of affinity-tag and affinity-ligand.

- 35 In a preferred aspect of the first embodiment, step d) further comprises the step of

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washing to remove unbound material, the amount of affinity medium-associated label being measured in an appropriate fashion (or the polymerase activity of the reconstituted RT being measured), wherein material bound to said affinity medium is proportional to the amount of labeled p51/p66 RT heterodimer.

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As will be readily recognized by persons skilled in the art, the sequence of events in the assay leading to the incubation of p66 monomer and p51 can be modified with the same outcome. For example: the p51 RT subunit can be immobilized to a solid-phase prior to its incubation with p66 subunit. Similarly, the dilution of buffer promoting subunit association can take place at the same time as mixing of the p66 and p51 subunits or after the p66 solution has been contacted with p51.

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Similarly, the contacting of the test compound can be carried out in a different sequence without affecting the principle of the assay. For example, the test compound can be added to the p66 homodimer solution prior to addition of the dissociation agent; prior to the mixing with the p51 subunit; prior to the dilution with the re-association buffer; or at the same time as dilution with the re-association buffer.

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EXAMPLES**Materials and methods****1. RT expression plasmids**

The gene for the 51 kDa subunit of HIV-1 RT (HXB2 strain) was cloned into the pBAD HisB prokaryotic expression system (Invitrogen) between the *XhoI* and *HindIII* restriction sites, to give pBAD-HisRT51. This construct allows for the arabinose-inducible expression of the p51 subunit of RT as an N-terminal polyhistidine (6xHis) fusion protein following transformation of an appropriate bacterial strain (e.g., *E. coli* JM109).

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To provide for specific labeling of the 66 kDa RT subunit with reagents allowing fluorometric detection, site-directed mutagenesis was carried out using Quick Change™ Site-directed Mutagenesis Kit (Stratagene) to produce P2C/C38S/C280S RT. This eliminated the two Cys residues of WT (Wild Type) RT (C38, C280) and inserted a single Cys residue near the N-terminus of the subunit.

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The genes for the WT and the P2C/C38S/C280S (**SEQ ID NO. 1**) 66 kDa subunit of HIV-1 RT were cloned into the pKK223-3 prokaryotic expression vector (Amersham Pharmacia Biotech) between the *Eco R1* and *Hind III* restriction sites, to yield pKK-RT66WT and pKK-RT66-P2C, respectively. These vectors provide for the IPTG-dependent expression of the non-HIS-tagged 66 kDa RT subunit.

2. Reagents for TRF assay of RT dimer formation

Delfia Eu-N1-iodoacetomido chelate and Eu³⁺ Standard, Delfia Enhancement solution and Delfia wash concentrate were purchased from Perkin Elmer.

Ultrapure urea, tris(hydroxymethyl)aminomethane and water were acquired from Fluka. Magnesium chloride (SigmaUltra) and diethylenetriamine-pentaacetic acid (DTPA) were from Sigma.

96-well Ni-NTA HisSorb plates (white) were purchased from Qiagen or Pierce. Fluorescence measurements were made with a SpectraMAX GeminiXS microplate fluorometer (Molecular Devices).

3. Expression and purification of RT p51 subunit monomer and the RT p66/p66 homodimer

E. coli JM109 transformed with pBAD-HisRT51 were grown at 37°C in "Terrific broth" to mid-log phase (OD₆₀₀ of 0.5). Expression of p51 RT was then induced by the addition of 1% arabinose. After further incubation for 3h, cells were harvested by centrifugation and lysed using BugBuster™ protein extraction reagent (Novagen).

The p51 RT was purified from the lysate by Ni²⁺-NTA affinity chromatography, using His-Bind Resin (Novagen). Both lysis and extraction were carried out according to the manufacturer's specifications. Following elution from Ni²⁺-NTA resin, p51 RT was buffer-exchanged into 50mM Tris-HCl (pH 7.5, 4°C) containing 60mM MgCl₂ and 50% glycerol, by two passages through NAP™25 column (Amersham Pharmacia Biotech). The final protein sample was stored in aliquots at -80°C. Purified p51 RT exists predominantly as a monomer.

Expression of WT and mutant p66 RT was induced by the addition of 1mM IPTG to 1L of appropriately transformed mid-log phase *E. coli* JM109 cells (grown in "Terrific broth" at 37°C). Following addition of IPTG, growth of the bacteria was continued at

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32°C for an additional 5 hours. Cells were harvested by centrifugation. Purification of the p66 RT was carried out as previously described (Fletcher *et al.*, 1996, "Single step purification of HIV-1 recombinant wild type and mutant reverse transcriptase" *Protein Expression & Purification* 7, 27-32). Purified p66 RT exists predominantly as a homodimer.

4. Alkylation of P2C/C38S/C280S p66/p66 RT with the Eu³⁺-N1-iodoacetamido chelate

- 10 Purified P2C/C38S/C280S p66/p66 RT was concentrated to approximately 20 μ M (~2.5mg/ml) in 50 mM Tris-HCl buffer (pH7.9 at 20°C) containing 100 mM NaCl and 1 mM TCEP-HCl (Tris(2-carboxyethyl)phosphine hydrochloride (Pierce)) at room temperature. An aliquot of Eu-N1-iodoacetamido chelate protein-modification reagent (prepared as a 5 mM stock solution in DMSO) was added to provide an
- 15 approximately 20-fold molar excess relative to the p66 subunit concentration. The alkylation reaction was allowed to proceed for up to 6 hours at room temperature. Alternatively, the reaction may be carried out overnight at 4°C. Upon completion of the reaction, an excess of DTT was added to consume unreacted Eu-N1-iodoacetamido chelate. The alkylated p66/p66 RT was separated from the DTT-reagent conjugate by two passages through a NAPTM25 column equilibrated with a
- 20 25 mM Tris-HCl buffer (pH 7.5, 20°C) containing 60mM MgCl₂ and 50% glycerol. The stoichiometry of labeling was generally found to be between 0.7 – 1 mol of Eu-N1-iodoacetamide per mol of p66 RT subunit (i.e., 1.5 – 2 mol/mol p66/p66 RT homodimer).
- 25
- Kinetic characterization of the mutant and the chemically modified RT proteins indicated that the P2C/C38S/C280S p66/p66 RT possessed comparable RNA-dependent DNA polymerase (RDDP) specific activity to the wt p66/p66 homodimer enzyme (data not shown). The P2C/C38S/C280S p66/p66 RT chemically alkylated
- 30 with the Eu-N1-iodoacetamido chelate retains approximately 80% of the RDDP activity of the unmodified protein (Table 1). In addition, the modified RT is equally sensitive to inhibition by TSAOe³T (Table 2) as the unmodified P2C/C38S/C280S p66/p66 mutant, which in turn is equally sensitive as WT p66/p66 RT (data not shown). Thus, the genetic and chemical manipulations of RT required to prepare
- 35 reagents for the dimerization assay appear to have little effect on the response of the

enzyme within the parameters of the assay protocol.

Table 1. RNA-dependent DNA polymerase (RDDP) and DNA-dependent DNA polymerase (DDDP) specific activity of P2C/C38S/C280S p66/p66 RT.

Polymerase substrate	% activity relative to unlabeled p66/p66
	Eu ³⁺ -labeled p66/p66
Poly rC:dG	80
Poly rA:dT	77
Poly dC:dG	73

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Table 2. Inhibition of P2C/C38S/C280S p66/p66 RT by HIV RT inhibitors.

Inhibitor	IC₅₀ (μM)	
	unlabeled p66/p66	Eu³⁺-labeled p66/p66
Nevirapine	1	1
TSAOe3T	3	3

5. Microplate assay to monitor p66-p51 RT dimerization

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The general principle of the assay involves mixing of His6x-p51 with fluorophore-labeled p66 RT in solution, with subsequent solid-phase capture of resulting His6x-p51/ fluorophore-labeled p66 RT heterodimer on Ni²⁺-NTA microplates. After washing to remove unbound material, the amount of microplate-associated

15 fluorescence is measured in an appropriate fluorescence plate reader. Alternatively, the polymerase activity of the reconstituted RT can be determined by using a fluorescent RNA/DNA intercalator such as PicoGreen™.

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While purified recombinant p51 RT exists as a monomer, purified p66 RT is predominantly dimeric. Since the subunit association energy of the p66/p66 RT homodimer is only minimally less than that of the p51/p66 RT heterodimer, the formation of the heterodimer following simple mixing of p51 and p66/p66 RT would be too slow to be convenient in a high throughput assay. Accordingly, a search was carried out to determine the optimal type and concentration of denaturant needed to

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provide dissociation of the p66/p66 homodimer without significant effect on protein secondary structure. Urea was found to be superior to guanidinium chloride as denaturant. The effect of urea concentration on p66/p66 dissociation and over protein denaturation (change in protein secondary structure) is shown in Figure 1.

- 5 Optimization of the urea concentration for maximization of the TRF signal in the dimerization assay system is given in Figure 3.

Urea at 1-3.5M was found to be optimal, based on a variety of data, including those illustrated in Figures 1, 2 and 3. There are a large number of tryptophan residues at the subunit interface of HIV-1 RT; these are essentially "buried" and therefore fluoresce at about 335 nm when RT is excited at wavelengths between 280 – 295 nm. As can be seen in Figure 1A, these residues become increasingly "solvent exposed" with increasing urea concentrations, as indicated by the red-shift in $\lambda_{\text{max}}^{\text{em}}$ to 350 nm. At 3.5M urea, this red-shift is approximately half-maximal. At the same time, however, this concentration of urea has little effect on the molar ellipticity of RT (Figure 1B), indicating that the protein secondary structure may not be significantly altered (i.e., no significant "denaturation" of the protein has occurred). This suggests that the increased solvent exposure of the protein tryptophan residues arises primarily from dissociation of the RT subunits. The changes in ANS binding to RT as a function of urea concentration (Figure 1C) are consistent with this interpretation.

Figure 2 shows the change in the dimerization state of p66/p66 RT as a function of urea as determined by size-exclusion chromatography. In this case, dissociation of the homodimer appears essentially complete at 2M urea and a progressive denaturation/aggregation of the p66 monomer appears to take place as the peak slowly shifts toward higher molecular weights with increasing urea concentrations. Figure 3 shows the signal obtained in the time-resolved RT subunit reassociation assay when labeled RT p66/p66 homodimer and His-tagged p51 monomer were incubated in urea concentrations between 0 – 6 M, prior to addition of the mixture to the Nickel-coated microplates. Thus, 1-3.5M urea was selected as the concentration range likely to provide the highest level of re-association with the least detrimental effect on RT secondary structure.

Additional experiments show that the use of 50 mM Na_2SO_4 rather than 100 mM MgCl_2 in the renaturation buffer significantly enhances the TRF specific signal to

background in the assay (Table 3).

Table 3. Signal to noise ratio in the RT dimerization assay as a function of renaturation buffer salt composition.

Label on p66 RT	Salt	S/N
Tetramethyl rhodamine	100 mM MgCl ₂	2.0
Oregon Green	100 mM MgCl ₂	3.2
Eu-N1-iodoacetamide (TRF)	100 mM MgCl ₂	6.5
Eu-N1-iodoacetamido (TRF)	50 mM Na ₂ SO ₄	10.8

6. Antiviral activity of RT subunit disrupters and RT subunit association inhibitors.

Assay Protocol

- 10 The concept of the assay is shown in Figure 5A. In a total volume of 20 μ l, mix 100 pmol of purified His6x-p51 RT subunit and 200 pmol of Eu-N1-iodoacetamido chelate labeled p66 RT subunit (i.e., 100 pmol RT p66/p66 homodimer) with urea (prepared in 25 mM Tris-HCl pH 7.5, 20°C, containing 100 mM MgCl₂) to provide a final concentration of 3.5 M urea. Incubate this mixture for 1 h at room temperature.

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Dilute this solution into 200 μ L of a solution of the compound of interest at 10 μ M (in 25 mM Tris-HCl pH7.5, 20°C, containing 50 mM Na₂SO₄) contained in a well of a 96-well Ni-NTA HiSorb Plate.

- 20 The dilution step provides a 10-fold dilution to reduce the urea concentration from 3.5M to 0.35M, thereby allowing formation of the RT p66/p51 heterodimer. Dimer formation is allowed to proceed for 6 h, then the plates are washed 3x with Delfia wash buffer containing 200 μ M DTPA. After washing, 200 μ L of the Delfia enhancement solution is added to each well, followed by incubation at room
- 25 temperature for 30 minutes. Fluorescence measurements are made using a Molecular Devices SpectraMAX GeminiXS instrument, using excitation, emission and cutoff wavelengths of 335nm, 615nm and 530nm, respectively. Using this format, TSAOm³T decreases the signal in the assay of the invention (Figure 5B). TSAOm³T is part of the TSAO family and is thought to inhibit RT dimerization to a

similar degree as TSAOe³T. In addition, nevirapine and efavirenz are two non-nucleoside reverse transcriptase inhibitors that do not inhibit the enzyme *via* RT dimerization. In fact, it has been reported that these two NNRT inhibitors are chemical enhancers of dimerization of the HIV-1 RT causing inhibition of the polymerase activity through deleterious conformational changes (Tachedjian *et al.*,
5 **2001**, PNAS, 7188-7193). In this particular assay format, the % signal relative to control presented in Figure 5B is supportive of this hypothesis and supports the usefulness of the present assay to identify inhibitors / enhancers of the HIV RT dimerization process.

Alternatively, the polymerase activity of the reconstituted p66/p51 reverse transcriptase can be measured. In this format, the necessary substrates (template primer, nucleotide, magnesium chloride) in an appropriate buffer (Tris pH 7.8 containing DTT, GSH & Chaps) are added directly to the wells following the
15 dimerization step and incubated for 1 hour after which time the amount of polymerization is determined by addition of a fluorescent RNA/DNA intercalator. An example is as follows: The homodimer p66/p66 (10-20 pmols) is incubated in the presence of HIS-p51 (25-50 pmols) in 1 M urea (prepared in 20 mM Tris-HCl pH 8.0 containing 250 mM NaCl) in a final volume of 10 μ L for 1 hour to allow dissociation of
20 the homodimer. Then an initial 5-fold dilution (final volume of 50 μ L) in re-association buffer (50 mM Tris-acetate, pH 8.0, 500 mM NaCl, 0.05% Tween-20, 0.01% BSA) with a test compound in the association buffer, is performed for 1-2 hours to allow for heterodimer formation by reduction of the urea concentration to 200 mM. The final step consists of capturing the reconstituted His-tagged
25 heterodimers by transferring the sample in the wells of a nickel plate containing 100 μ L of re-association buffer and incubating for 1 hour. After 3 washing cycles, a polymerase assay cocktail is added and the reaction is allowed to proceed for 1-2 hours at 37⁰ C before the elongated RNA/DNA products are detected with the fluorescent intercalator PicoGreen™.

7. Alternative format of the assay

The biologically relevant and active form of HIV RT found in infectious virions is a heterodimer containing two polypeptides, p66 and p51; the latter derived from the
35 former by proteolytic cleavage of its C-terminal domain by HIV protease during viral

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maturation. The two subunits of 66 and 51 kDa form are present in a 1 to 1 ratio. This heterodimeric RT is believed to be produced in a two-step dimerization process, the kinetics of which involve the rapid association of the p66 and p51 subunits into an immature dimer, followed by a slow conformational change yielding the fully
5 active form ($p66 + p51 \rightarrow p66/p51 \text{ immature} \rightarrow p66/p51 \text{ active}$; Divita et al., **1995**, J. Mol. Biol. 245, 508-52112, 13).

However, more recent evidence has also suggested an alternate mechanism for the formation of RT which first involves homodimerization of the p66 subunits into a
10 p66/p66 homodimer followed by HIV protease cleavage into a p66/p51 heterodimer (Oral presentation, Retrovirus 2001 Cold Spring Harbor, NY, May 26, 2001). It is unclear at this time which mechanism prevails during viral replication and screening assays designed to probe each pathway individually may offer new therapeutic approaches. It is therefore another aspect of this invention to probe for compound
15 that can potentially interfere with the homodimerization of p66 subunits.

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CLAIMS

1. A method for measuring heterodimerization of HIV RT, which comprises the steps of:
 - a) providing a first solution comprising p66 subunit homodimers in the presence of a dissociation agent;
 - 5 b) contacting said first solution with p51 RT subunits and incubating in the presence of a reassociation buffer to allow association of a reconstituted RT complex of p66/p51 RT subunits, wherein one of said subunits comprises an affinity tag and the other of said subunits comprises a detectable label;
 - 10 c) contacting the incubate of step b) with an affinity medium under conditions that enable the p66/p51 complex to bind to said affinity medium; and
 - d) determining the amount of complex formed by measuring the level of detectable label bound to the affinity medium
2. The method according to claim 1, wherein in step d), said amount of complex is determined by measuring the polymerase activity of said reconstituted RT complex.
3. The method, according to claim 1, wherein the first solution in step a) comprises a buffer selected from Tris-HCl, HEPES or bis-Tris.
- 15 4. The method, according to claim 2, wherein the buffer is Tris-HCl at a concentration of between 0mM and 50mM.
5. The method, according to claim 1, wherein the first solution in step a) further comprises a salt selected from Na₂SO₄ or MgCl₂.
6. The method, according to claim 5, wherein the salt is MgCl₂ at a concentration of between 0mM and 100mM.
7. The method, according to claim 1, wherein in step a), the dissociation agent is selected from urea, guanidinium-HCl or acetonitrile.
8. The method, according to claim 7, wherein the dissociation agent is urea at a

concentration from 1 to 3.5 M.

9. The method, according to claim 1, wherein the re-association buffer in step b) is the same buffer used to dissociate the p66/p66 subunits, said re-association buffer being denaturant-free.

10. The method, according to claim 1, wherein the re-association buffer is present in excess amount to the denaturant buffer to dilute the dissociation agent sufficiently to allow re-association of p66 and p51 subunits.

11. The method, according to claim 10, wherein the re-association buffer is added in a 10-fold excess over the dissociation buffer.

12. The method, according to claim 1, wherein the re-association buffer in step b) further comprises a salt selected from Na_2SO_4 or MgCl_2 .

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13. The method, according to claim 12, wherein the salt is Na_2SO_4 at a concentration of between 0mM and 100mM.

14. The method, according to claim 13, wherein the salt is Na_2SO_4 at a concentration of 50mM.

15. The method, according to claim 1, wherein the p51 in step b) is affinity-tagged or has a detectable label.

16. The method, according to claim 15, wherein the p51 in step b) is affinity-tagged.

17. The method, according to claim 1, wherein the p66 in step b) has a detectable label or is affinity-tagged.

10

18. The method, according to claim 17, wherein the p66 in step b) has a detectable label.

19. The method, according to claims 15 or 17, wherein the affinity-tag in step b)

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is selected from the group consisting of: a hexa-histidine tag, FLAG, T7, HA, GST, and biotin.

20. The method, according to claim 19, wherein the affinity tag is hexa-histidine.

21. The method, according to claims 15 or 17, wherein the detectable label is selected from the group consisting of: a fluorescent label, a radioactive atom, a chemiluminescent label, a detectable antibody specific to p66 or specific to a tag attached to p66, and an enzymatic marker.

22. The method, according to claim 21, wherein the detectable label is a fluorescent label selected from the group consisting of: fluorescein, Oregon green, rhodamine, Texas-red, phycoerythrin and Eu^{3+} .

23. The method, according to claim 22, wherein the fluorescent label is Eu^{3+} .

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24. The method, according to claim 1, wherein the detectable label in step b) is added to a cysteine residue that is introduced recombinantly in place of a proline residue at the N-terminus of p66.

25. The method, according to claim 1, wherein the affinity medium in step c) captures any reconstituted labeled-p66/tag-p51/ RT heterodimer.

26. The method, according to claim 1, wherein in step c), the affinity medium is a solid phase coated with a receptor that is suitable to capture the affinity tag.

27. The method, according to claim 26, wherein the solid phase is coated with a receptor selected from the group consisting of: Ni^{2+} , anti-tag antibodies, glutathione and streptavidin.

28. The method, according to claim 27, wherein solid phase is coated with Ni^{2+} .

10

29. The method, according to claim 26, wherein the affinity tag is histidine.

- 30.** The method, according to claim 26, wherein the solid phase is a microplate.
- 31.** The method, according to claim 1, wherein step d) further comprises the step of washing to remove unbound material, the amount of affinity medium-associated label being measured in an appropriate fashion (or the polymerase activity of the reconstituted RT being measured), wherein material bound to said affinity medium is proportional to the amount of labeled p51/p66 RT heterodimer.
- 32.** A method for identifying compounds capable of modulating the HIV RT heterodimerization, comprising:
- carrying out steps a) to d), according to claim 1, in the presence or absence of a test compound; and
 - 5 e) comparing the test compound sample to a control sample lacking said compound, whereby modulated p66/p51 complex formation in the test compound sample is indicative of the ability of said compound to modulate heterodimerization.
- 33.** A method for identifying compounds capable of interfering with the HIV RT heterodimerization, comprising:
- 10 carrying out steps a) to d), according to claim 1, in the presence or absence of a test compound; and
 - e) comparing the test compound sample to a control sample lacking said compound, whereby decreased p66/p51 complex formation in the test compound sample is indicative of the ability of said compound to inhibit
 - 15 heterodimerization.
- 34.** A method for identifying compounds capable of enhancing the HIV RT heterodimerization, comprising:
- carrying out steps a) to d), according to claim 1, in the presence or absence of a test compound; and
 - 20 e) comparing the test compound sample to a control sample lacking said compound, whereby increased p66/p51 complex formation in the test compound sample is indicative of the ability of said compound to enhance heterodimerization.

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- 35.** A method for measuring homodimerization of HIV RT, which comprises the steps of:
- a) providing a first solution comprising first p66 subunit homodimers in the presence of a dissociation agent;
 - b) contacting said first solution with second p66 subunits homodimers, in the presence of said dissociation agent, and incubating in the presence of a reassociation buffer to allow association of a complex of p66/p66 RT subunits, wherein one of said subunits comprises an affinity tag and the other of said subunits comprises a detectable label;
 - c) contacting the incubate of step b) with an affinity medium under conditions that enable the p66/p66 complex to bind to said affinity medium; and
 - d) determining the amount of complex formed by measuring the level of detectable label bound to the affinity medium (or by measuring the reconstituted RT polymerase activity).
- 36.** A method for identifying compounds capable of modulating the HIV RT homodimerization, comprising:
- carrying out steps a) to d), according to claim 35, in the presence or absence of a test compound; and
 - e) comparing the test compound sample to a control sample lacking said compound, whereby modulated p66/p66 complex formation in the test compound sample is indicative of the ability of said compound to modulate homodimerization.
- 37.** A method for identifying compounds capable of interfering with the HIV RT homodimerization, comprising:
- carrying out steps a) to d), according to claim 35, in the presence or absence of a test compound; and
 - e) comparing the test compound sample to a control sample lacking said compound, whereby decreased p66/p66 complex formation in the test compound sample is indicative of the ability of said compound to inhibit homodimerization.

24

38. A method for identifying compounds capable of enhancing the HIV RT homodimerization, comprising:

carrying out steps a) to d), according to claim 35, in the presence or absence of a test compound; and

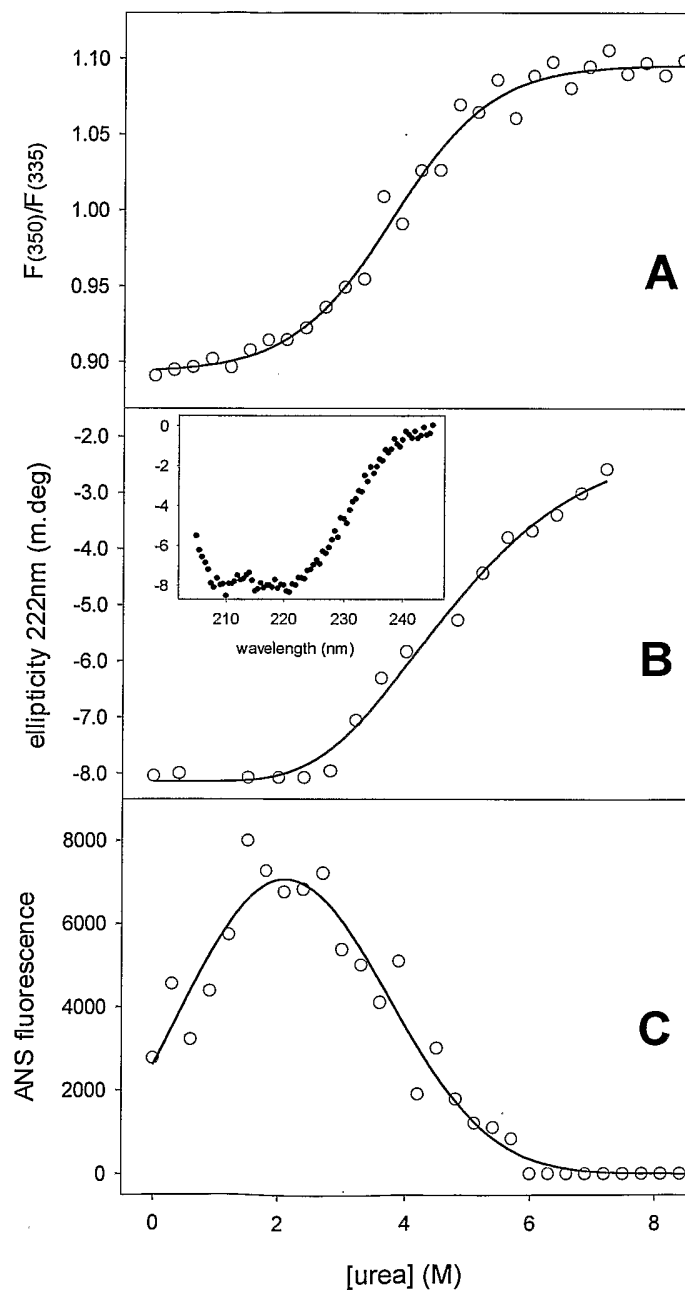
5 e) comparing the test compound sample to a control sample lacking said compound, whereby increased p66/p66 complex formation in the test compound sample is indicative of the ability of said compound to enhance homodimerization.

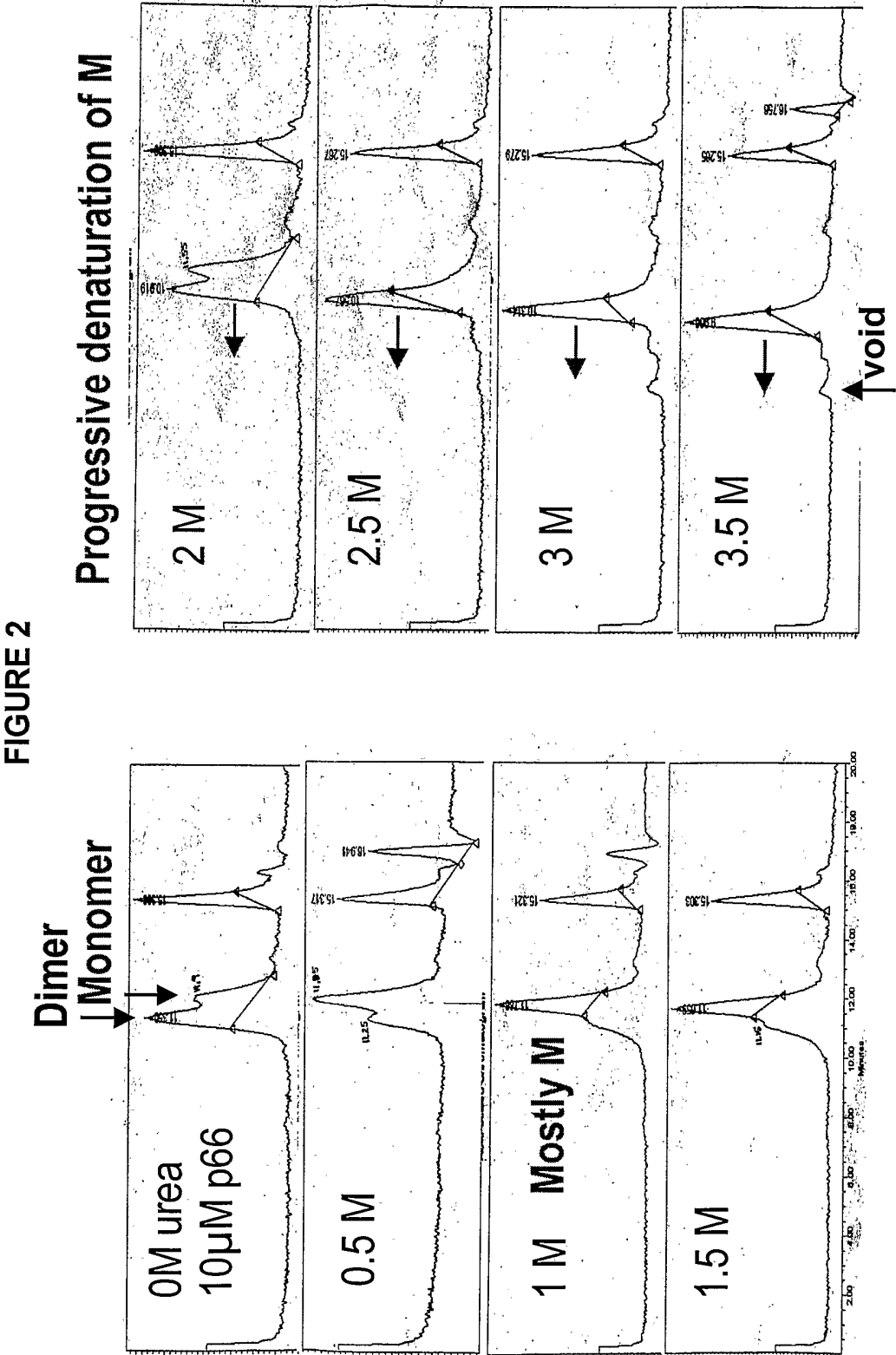
39. A kit for testing compounds potentially modulating the HIV RT heterodimerization, said kit comprising a plurality of affinity-tagged p66 subunit homodimers, a plurality of labeled p51 RT subunits, a dissociation agent, a reassociation buffer, an affinity medium and instructions on how to use said subunits for identifying test compounds binding to said transcriptase.

40. A kit for testing compounds potentially modulating the HIV RT homodimerization, said kit comprising a plurality of affinity tagged p66 subunit homodimers, a plurality of labeled p66 RT subunits, a dissociation agent, a reassociation buffer, an affinity medium and instructions on how to use said subunits for identifying test compounds binding to said transcriptase.

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FIGURE 1





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FIGURE 3

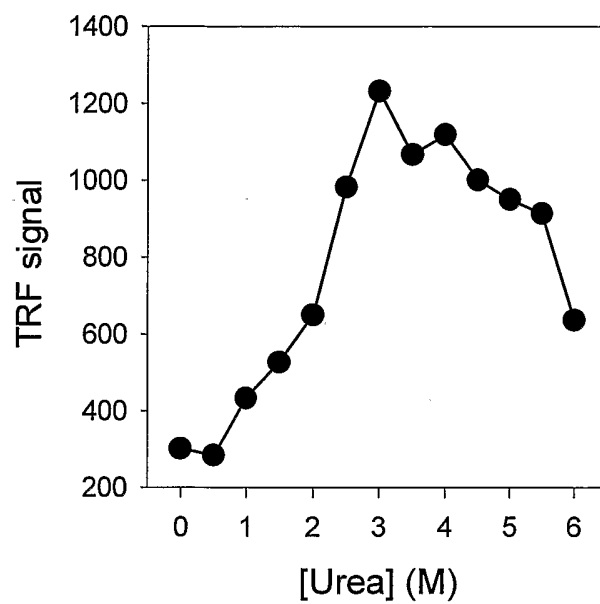
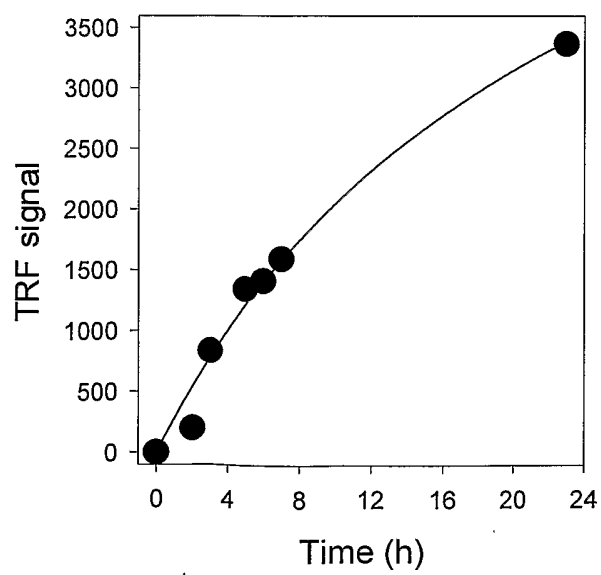


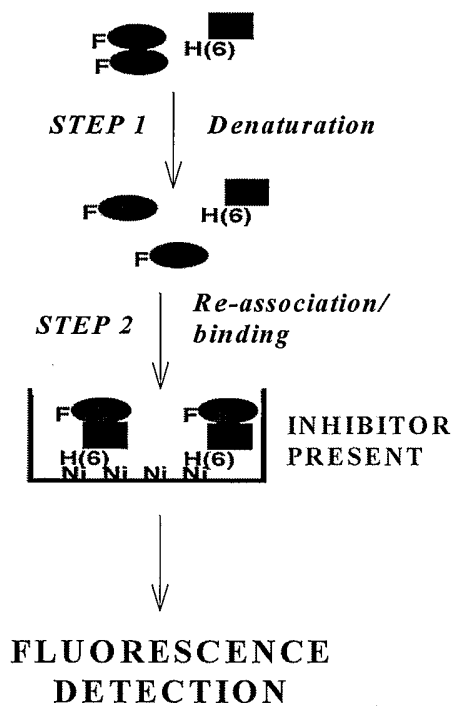
FIGURE 4



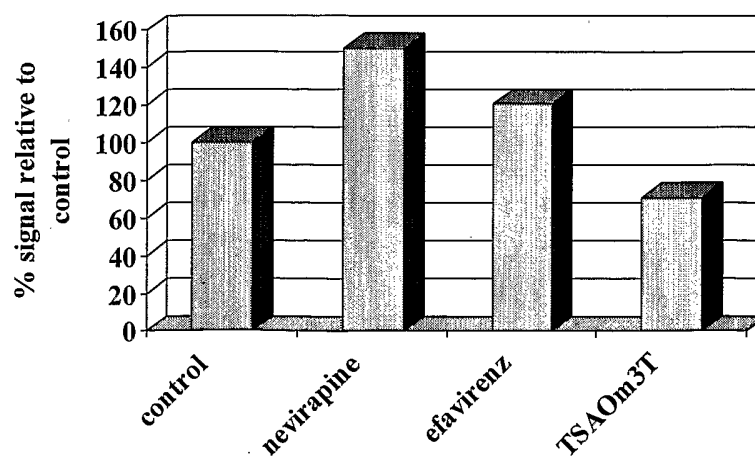
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FIGURE 5

A.



B.



SEQUENCE LISTING

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RT Dimerization

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Lys	Ile	Gly	Pro	Glu	Asn	Pro	Tyr	Asn	Thr	Pro	Val	Phe	Ala	Ile	Lys
Lys	Lys	Asp	Ser	Thr	Lys	Trp	Arg	Lys	Leu	Val	Asp	Phe	Arg	Glu	Leu
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Thr	Asp	Ser	Gln	Tyr	Ala	Leu	Gly	Ile	Ile	Gln	Ala	Gln	Pro	Asp	Gln
			500					505					510		
Ser	Glu	Ser	Glu	Leu	Val	Asn	Gln	Ile	Ile	Glu	Gln	Leu	Ile	Lys	Lys
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Asn	Glu	Gln	Val	Asp	Lys	Leu	Val	Ser	Ala	Gly	Ile	Arg	Lys	Val	Leu
545					550					555					560