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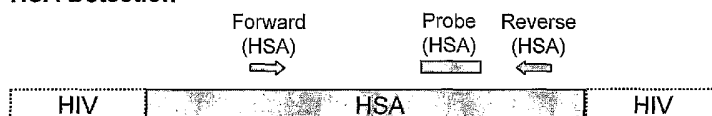
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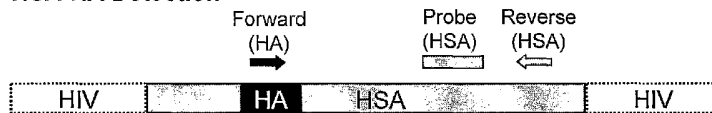
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(54) Title: REPORTER CONSTRUCTS AND VIRAL FITNESS ASSAYS

HSA Detection



HSA-HA Detection



(57) Abstract: The invention provides a novel reporter nucleic acid that can be used in viral detection assays, and methods for making and using same. In one aspect, the invention provides reporter constructs comprising heat stable antigen (HSA) reporter sequences comprising detectable heterologous (non-HSA) marker sequences that can be used in viral detection and viral growth assays (e.g., viral competition assays), and methods for making and using same. In one aspect, the invention provides methods for determining the relative fitness of at least two viruses, e.g., under a selective constraint or a particular growth condition, including determining differences in growth rate of the different viruses under the constraints of an immune response, a drug, a vaccine or a particular set of environmental conditions. Thus, the invention provides compositions and methods to determine the relative viral fitness (i.e., the ability of a given virus to replicate) of two or more viruses.

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REPORTER CONSTRUCTS AND VIRAL FITNESS ASSAYS

Cross-Reference to Related Applications

This application claims benefit of U.S. provisional patent application 60/607,893 filed September 7, 2004. The contents of this document are expressly incorporated herein by
5 reference in its entirety for all purposes.

Statement of Rights to Inventions Made Under Federally Sponsored Research

This invention was supported by Grant Nos. AI043203 and AI051970 of the National Institutes of Health. The United States government may have certain rights in this invention.

FIELD OF THE INVENTION

This invention relates to molecular and cellular biology, molecular genetics, viral diagnostics and gene therapy. In one aspect, the invention provides reporters constructs comprising heat stable antigen (HSA) reporter sequences comprising detectable heterologous (non-HSA) marker sequences that can be used in viral detection assays, and methods for making
15 and using same. In one aspect, the invention provides methods (e.g., viral competition assays) for determining the relative fitness of at least two viruses, e.g., under a selective constraint or a particular growth condition, including determining differences in growth rate of the different viruses under the constraints of an immune response, a drug, a vaccine or a particular set of environmental conditions. Thus, the invention provides compositions and methods to determine
20 the relative viral fitness (i.e., the ability of a given virus to replicate) of two or more viruses.

BACKGROUND

The relative ability of a given virus or virus mutant to replicate is termed viral fitness. Fitness is dependent on both viral and host factors, including the genetic composition of the virus, the host immune response, and selective pressures such as the presence of anti-viral
25 compounds. Many drug-resistant variants of HIV-1 are less fit than the wild-type, i.e. they grow more slowly in the absence of drug selection. However, since the replication of the wild-type virus is inhibited in the presence of drug, the resistant mutant can outgrow it. The reduction in fitness may be a result of several factors including: decreased ability of the mutated enzyme (e.g., reverse transcriptase) to recognize its natural substrates, decreased stability of the mutant
30 protein, or decreased kinetics of enzymatic catalysis. See Back et al., EMBO J. 15: 4040-4049,

1996; Goudsmit et al., J. Virol. 70: 5662-5664, 1996; Maschera et al., J. Biol. Chem. 271: 33231-33235, 1996; Croteau et al., J. Virol. 71: 1089-1096, 1997; Zennou et al., J. Virol. 72: 300-3306, 1998; Harrigan et al., J. Virol. 72: 3773-3778, 1998; Kosalaraksa et al., J. Virol. 73: 5356-5363, 1999; Gerondelis et al., J. Virol. 73: 5803-5813, 1999. Drug resistant viruses that are less fit than wild type may be less virulent i.e. they may cause damage to the host immune system more slowly than a wild type virus. Immunological decline may be delayed after the emergence of drug resistant mutants, compared to the rate of immunological decline in an untreated patient. The defect causing reductions in fitness may be partially or completely compensated for by the selection of viruses with additional amino acid substitutions in the same protein that bears the drug resistance mutations (for example, see Martinez-Picado et al., J. Virol. 73:3744-3752, 1999), or in other proteins which interact with the mutated enzyme. Thus, amino acids surrounding the protease cleavage site in the gag protein may be altered so that the site is better recognized by a drug-resistant protease enzyme (Doyon et al., J. Virol. 70: 3763-3769, 1996; Zhang et al., J. Virol. 71: 6662-6670, 1997; Mammano et al., J. Virol. 72: 7632-7637, 1998).

SUMMARY

The invention provides chimeric nucleic acid constructs comprising a nucleotide sequence encoding a heat stable antigen (HSA) reporter sequence comprising a detectable heterologous (non-HSA) marker sequence, wherein the detectable heterologous marker sequence is disposed within or in place of sequence encoding all or part of the extracellular domain of the heat stable antigen (HSA) reporter sequence. In one aspect, the reporter sequence encodes a human or a murine heat stable antigen (HSA) surface protein, e.g., CD24 (see, e.g., UniProtKB/Swiss-Prot primary accession no. P24807). The detectable heterologous sequence can encode an epitope recognized by an antibody, including e.g., single stranded antibodies, CDRs, antigen binding sites and the like. In one aspect (optionally), the epitope (specifically bound by the antibody, thus making the heterologous detectable) is derived from a viral polypeptide or peptide, e.g., an influenza hemagglutinin (HA) or related polypeptide. In one aspect (optionally), the epitope replaces an HSA sequence (e.g., all or part of an extracellular domain of HSA), e.g., replaces an antibody epitope in HSA, allowing separate antibody-based detection of both HSA and HA.

In one aspect, a chimeric nucleic acid construct of the invention, e.g., a nucleotide sequence encoding a heat stable antigen (HSA) reporter sequence comprising a detectable heterologous (non-HSA) marker sequence, is inserted into any region of a virus; and, depending

on the particular virus and the particular set of conditions to be used in a method of the invention, e.g., growth competition assays comprising challenging at least two viruses (e.g., at least one recombinant virus of the invention) with, e.g., lymphocytes such as CTLs, an anti-viral drug, the chimeric nucleic acid can be inserted into any region of the virus with or without deletion of a viral sequence.

In one aspect, the size of the inserted sequence corresponds to the size of the sequence deleted from the virus. For example, in one aspect the inserted sequence (e.g., a reporter sequence, or chimeric construct of the invention) is the same size or substantially the same size as a segment deleted from the virus, e.g., an HIV, e.g., HIV-1, for example, a *nef* or a *vpr*. In one aspect, the inserted sequence (e.g., a reporter sequence, or chimeric construct of the invention) is the same size or substantially the same size, or smaller than, the sequence deleted from a virus "parent", e.g., an HIV genome. For example, in one aspect, the invention provides recombinant viruses wherein sequences non-essential or relatively non-essential to the "fitness" of the virus are deleted (e.g., HIV-1 *nef* or HIV-1 *vpr*) and replaced in whole or in part by a chimeric construct of the invention, e.g., comprising a heterologous detectable sequence, e.g., an epitope detectable by an antibody, or, a sequence that can be specifically bound by an amplification primer such that each recombinant virus of a growth competition assay of the invention can be individually detected and measured, e.g., by PCR, such as "real time" PCR. Depending on the particular conditions to be used in practicing the methods of the invention, any portion of a virus can be partially or completely deleted, e.g., a *nef* or a *vpr*. However, in one aspect, a chimeric nucleic acid of the invention can be inserted into any part of a virus without deletion of a viral sequence.

The invention provides nucleic acid, e.g., an isolated, recombinant or synthetic nucleic acid, comprising a chimeric sequence as set forth in SEQ ID NO:1 (Vpr-HSA-HA) or SEQ ID NO:21 (Nef-HSA-HA), including, for example, recombinant viruses. The invention also provides chimeric polypeptides encoded by the chimeric nucleic acid constructs or recombinant viruses of the invention, e.g., encoded by SEQ ID NO:1 (Vpr-HSA-HA) or SEQ ID NO:21 (Nef-HSA-HA).

The invention provides recombinant viruses comprising a chimeric nucleic acid construct of the invention, wherein in one aspect (optionally) a sequence of the virus is deleted, and the chimeric nucleic acid construct is inserted into all, substantially all, or part of the deleted region of the virus. The virus can be any virus, e.g., a lentivirus, e.g., in one aspect (optionally), the lentivirus is a human immunodeficiency virus, such as a human immunodeficiency virus

type 1 (HIV-1) or HIV-2. In one aspect, a chimeric nucleic acid of the invention can be inserted into any part of a virus without deletion of a viral sequence.

In one aspect, the deleted region of the recombinant virus comprises (i) a reading frame of a non-essential gene or a gene that does not effect (or does not substantially effect, e.g., in a growth or viability context) the *in vivo* viability or replication (infectivity) of the virus, or (ii) a section of the viral genome that does not effect *in vivo* viability or replication (infectivity) of the virus. In one aspect, the chimeric nucleic acid construct of the invention is inserted into the region of the deleted region (of the recombinant virus) and/or in replacement for the deleted region of (i) or (ii). The entire non-essential gene region of the recombinant virus can be deleted and the chimeric nucleic acid construct can be inserted into and/or in replacement for the deleted region. The inserted chimeric nucleic acid construct can be substantially or exactly the same size as the deleted region. The deleted region of the recombinant virus can comprises all, substantially all or part of the deleted viral sequence, e.g., a deleted a *vpr* reading frame or a *nef* reading frame. In one aspect, the chimeric nucleic acid construct is inserted into and/or in replacement for all, substantially all or part of the *vpr* reading frame. In one aspect, all or substantially all of the *nef* or *vpr* region is deleted and the inserted chimeric nucleic acid construct is the same size or substantially the same size as the deleted sequence.

In one aspect, the heat stable antigen (HSA) reporter sequence in a chimeric nucleic acid construct of the invention (which can comprise a recombinant virus of the invention) encodes a murine heat stable antigen (HSA) lacking all, substantially all or part of its extracellular domain. The detectable heterologous marker sequence can encode an epitope specifically recognized by an antibody or other ligand, e.g., a soluble receptor, and the like. In one aspect, the epitope is derived from a viral polypeptide or peptide, and optionally the viral polypeptide comprises an influenza hemagglutinin (HA) polypeptide. The epitope can be any relatively small (i.e., no larger than the extracellular domain of a Vpr protein) epitope which specifically binds to an antibody, particularly an antibody that can specifically bind to the epitope with high specificity and/or high affinity.

The invention provides a recombinant virus comprising a heterologous sequence comprising a nucleic acid sequence as set forth in SEQ ID NO:1 (Vpr-HSA-HA) or SEQ ID NO:21 (Nef-HSA-HA), wherein the heterologous sequence is inserted into a deleted region of the virus, and the deleted region of the virus comprises (i) a reading frame of a non-essential gene or a gene that does not effect the *in vivo* viability or replication (infectivity) of the virus, or (ii) a section of the viral genome that does not effect *in vivo* viability or replication (infectivity)

of the virus. In one aspect, the virus comprises a human immunodeficiency virus and the deleted region comprises a *vpv* reading frame.

The invention provides methods for determining the relative fitness of at least two viruses, comprising: (a) introducing a first recombinant virus comprising a first reporter sequence into a host cell, and introducing at least a second recombinant virus comprising at least a second reporter sequence into the host cell, wherein each reporter sequence is distinguishably detectable from the other reporter sequences (wherein the host cell is permissive for growth of the virus); (b) culturing the host cell; (c) detecting the level of expression of the first and the at least second reporter sequences, wherein the expression of a reporter sequence is dependent on the fitness of the recombinant virus; and, (d) comparing the level of expression of the first and at least second reporter sequences, whereby a relatively higher expression of one reporter sequence over the other report sequence indicates greater fitness of that virus having the relatively higher expressed reporter sequence. In one aspect, the method provides for specific detection of the at least two viruses within any mixture, e.g., *in vitro* or *in vivo* system, such as a cell or cell lysate system.

In one aspect, at least one of the viruses used in any of the methods of the invention is a recombinant virus of the invention, or, alternatively, a chimeric nucleic acid construct of the invention can be used to practice a method of the invention.

The invention provides methods for determining the relative fitness of at least two viruses under a selective constraint or a particular growth condition, comprising: (a) introducing a first recombinant virus comprising a first reporter sequence into a host cell, and introducing at least a second recombinant virus comprising at least a second reporter sequence into the host cell, wherein each reporter sequence is distinguishably detectable from the other reporter sequences; (b) culturing the host cell in the presence or absence of a selective constraint, or in at least two different growth conditions; (c) detecting the level of expression of the first and the at least second reporter sequences, wherein the expression of a reporter sequence is dependent on the fitness of the recombinant virus; and (d) comparing the expression of the reporter sequences in the presence or absence of the selective constraint or growth condition, whereby the virus with the greatest fitness has an increased expression of its reporter sequence in the presence of the selective constraint or the particular growth condition.

In one aspect, the selective constraint comprises a virus-specific lymphocyte, e.g., T cells, either CD4 and/or CD8 T cells, or either cultured, cloned, subcloned, purified, isolated and/or specifically (e.g., stimulated by antigen) or non-specifically stimulated (e.g., by cytokine

and/or mitogen, e.g., Con A, PHA, and the like). In one aspect (optionally), the lymphocytes are specific for at least one version of the virus used in the assay, e.g., a wild type strain of the virus, a mutant, a substrain, a strain or a representative clade member of the virus. In one aspect (optionally), the lymphocytes are virus-specific or virus pre-stimulated T cells, e.g., either CD4 and/or CD8 T cells.

In one aspect, the selective constraint comprises an anti-viral agent, wherein in one aspect (optionally) the antiviral agent comprises an antiviral drug (e.g., a small molecule) or an antiviral antibody. The antiviral drug can be any anti-HIV drug, e.g., an experimental drug and/or a known drug (e.g., Truvada[®] (emtricitabine and tenofovir disoproxil fumarate), Agenerase[®] (amprenavir), Combivir[®] (combination of Retrovir[®] and Epivir[®]), Crixivan[®] (indinavir), Epivir[®] (3tc / lamivudine, - nucleoside analog reverse transcriptase inhibitor), Epzicom[®], Emtriva[®] (emtricitabine), Fortovase[®] (saquinavir), Fuzeon[®] (enfuvirtide), Retrovir[®], AZT (zidovudine), Sustiva[®] (efavirenz), Trizivir[®], Truvada[®] (Emtricitabine), Videx[®] (ddi / didanosine), Viracept[®] (nelfinavir), Viread[®] (tenofovir disoproxil fumarate), Zerit[®] (d4t / stavudine), Ziagen[®] (abacavir), and the like, or a combination thereof. For example, the growth competition assays of the invention can be used to determine what mutations develop in the course of a virus' developing a resistance to an experimental drug and/or resistance to a known drug, and/or what mutations develop in the course of a virus' exposure to an immune response (e.g., a virus-specific cytotoxic T cell, helper cell or mixed lymphocyte response), with or without concurrent exposure to a drug. Thus, in one aspect the invention is used to studying the impact of drug resistance mutations on viral, e.g., HIV, fitness. It is known that as HIV becomes resistant to some drugs, it pays a price in reduced fitness. This can result in clinical benefit even if the virus is not suppressed by the drugs. An exemplary assay can measure the fitness impact of mutations in any viral gene because it measures viral replication across the whole viral life cycle. For example, this aspect of the invention, this method, is particularly important as new drugs target other HIV genes besides *pol*, and the methods and compositions of the invention are used to develop and assess these new drugs, in addition to studying the effects of known drugs on viral epidemiology, e.g., the evolution of drug resistant strains, or pathology etiology.

In one aspect, infected cells are spiked with increasing concentrations of drugs; this aspect of the methods of the invention would demonstrate the impact of drugs on HIV replication or mutation ("forced evolution") in a controlled manner. In one aspect, infected cells are spiked with increasing amounts of lymphocytes, e.g., cytotoxic lymphocytes (CTL), mixed lymphocytes, peripheral blood mononuclear cells (PBMCs), and the like (see discussions,

below); this aspect of the methods of the invention would demonstrate the impact of lymphocytes (or a vaccine or an immune response) on HIV replication or mutation (“forced evolution”) in a controlled manner. In one aspect, infected cells are spiked with increasing concentrations of vaccine (e.g., antibody); this aspect of the methods of the invention would demonstrate the impact of a vaccine or an immune response on HIV replication or mutation (“forced evolution”) in a controlled manner. In one aspect, infected cells are spiked with increasing concentrations of a hormone or a cytokine; this aspect of the methods of the invention would demonstrate the impact of these immune modulators on HIV replication or mutation (“forced evolution”) in a controlled manner.

In one aspect, infected cells are exposed to varying amounts of a growth condition; this aspect of the methods of the invention would demonstrate the impact of these growth conditions on HIV replication or mutation (“forced evolution”) in a controlled manner. In alternative aspects of the methods of the invention, the at least two different growth conditions can comprises culturing the host cell in alternative *in vitro* culture environments, *in vivo* environments (including non-human animals, such as non-human primates or mice, such as human-SCID mice), temperatures and/or pHs.

The invention provides viral fitness assays based on direct growth competition of at least two viruses, e.g., at least two HIV-1 strains. In one aspect, the invention comprises use of molecular detection methodologies to measure the relative amount of the at least two competing viral strains in the growth competition assay, e.g., using amplification reactions, such as PCR. Thus, in one aspect, the methods of the invention use PCR primers that specifically bind different viruses of interest (e.g., competing viruses in a growth competition, or “fitness” assay), including specifically binding different viral mutants, viral strains, substrains, clades and the like, such that each recombinant virus can be independently detected. In one aspect, these PCR primers comprise the mutations of interest, or are primers custom-designed for the particular mutations of interest (such that each recombinant virus can be independently detected). In one aspect, the “paired reporter virus” strategy of the methods of the invention offer nearly identical strains of virus that allow comparison of isolated mutations with fixed primers. As demonstrated by the data set forth herein, this strategy is useful for replicative capacity measurements in viral competition assays.

The invention provides methods for determining the effectiveness of a viral vaccine, comprising: (a) introducing a first recombinant virus comprising a first reporter sequence into a host cell *in vitro* or *in vivo*, wherein the first virus is immune resistant, and

introducing at least a second recombinant virus comprising at least a second reporter sequence into the host cell *in vitro* or *in vivo*, wherein the at least a second virus is immune sensitive; (b) culturing the host cells *in vitro* or *in vivo* in the presence or absence of peripheral blood mononuclear cells (PBMC) isolated from a patient inoculated with a vaccine against the virus; (c) determining the level of expression of the first and the at least second reporter sequences, wherein the expression of each reporter sequence is dependent on the relative fitness of the recombinant virus; and (d) comparing the expression of the reporter sequences in the presence or absence of the PBMC, whereby the ratio of recombinant virus growth as indicated by reporter sequence expression reflects the effectiveness of the vaccine at eliciting a biologically relevant anti-viral immune response. In one aspect (optionally), the first and at least second recombinant virus are members of different strains, substrains or clades of the virus. In one aspect (optionally), the first and at least second recombinant virus are members of the same strain, substrain or clade of the virus, but differ in one or more residues of a nucleic acid sequence, protein sequence; and/or differ in viral protein expression (for example, if the mutation is in a promoter or an enhancer, or a temperature conditional mutation).

The invention provides methods for determining the relative effectiveness of a viral vaccine against at least two different viruses, the method comprising the steps of: (a) introducing a first recombinant virus comprising a first reporter sequence into a host cell *in vitro* or *in vivo*, and introducing at least a second recombinant virus comprising at least a second reporter sequence into the host cell *in vitro* or *in vivo*, (b) culturing the host cells *in vitro* or *in vivo* in the presence or absence of peripheral blood mononuclear cells (PBMC) or antibodies isolated from an individual inoculated with a vaccine directed against the virus; (c) determining the level of expression of the first and the at least second reporter sequences, wherein the expression of each reporter sequence is dependent on the relative fitness of the recombinant virus; and (d) comparing the expression of the reporter sequences in the presence or absence of the PBMC or antibodies, whereby the ratio of virus growth as indicated by reporter sequence expression reflects the effectiveness of the vaccine at eliciting a biologically relevant anti-viral immune response. In one aspect (optionally), the first and at least second recombinant virus are members of different genera, strains, substrains or clades of the virus. In one aspect (optionally), the first and at least second recombinant virus are members of the same genus, species, subspecies, strain, substrain or clade of the virus, but differ in nucleic acid sequence or viral protein expression.

The invention provides methods for determining the relative effectiveness of a test compound or an anti-viral drug against at least two different viruses, the method comprising the steps of: (a) providing at least one test compound or anti-viral drug; (b) providing at least two different recombinant viruses each comprising a detectably different reporter sequence, and
5 a host cell compatible with the growth of the at least two different recombinant viruses, (c) introducing a first recombinant virus comprising a first reporter sequence into the host cell, and introducing at least a second recombinant virus comprising at least a second reporter sequence into the host cell; (d) culturing the host cells in the presence or absence of the test compound or anti-viral drug; (e) determining the level of expression of the first and the at least second reporter
10 sequences, wherein the expression of each reporter sequence is dependent on the relative fitness of the recombinant virus; and (f) comparing the expression of the reporter sequences in the presence or absence of the test compound or anti-viral drug, whereby the ratio of virus growth as indicated by reporter sequence expression reflects the effectiveness of the test compound or anti-viral drug to elicit an anti-viral response against the first and at least second virus. In one aspect
15 (optionally), the first and at least second recombinant virus are members of different genera, species, subspecies, strains, substrains or clades of the virus. In one aspect (optionally), the first and at least second recombinant virus are members of the same genus, species, subspecies, strain, substrain or clade of the virus, but differ in nucleic acid sequence or viral protein expression.

20 In one aspect of any of the methods of the invention, the first and or at least second recombinant virus comprises a recombinant virus of the invention, e.g., a recombinant virus comprising a chimeric nucleic acid construct of the invention. The first reporter sequence can comprise a chimeric a heat stable antigen (HSA) reporter sequence and the at least second reporter sequence comprises a heat stable antigen (HSA) reporter sequence; and optionally the
25 first reporter sequence comprises SEQ ID NO:1 (HSA-HA) and the at least second reporter sequence comprises an HSA.

In one aspect of any of the methods of the invention, the first or the at least second reporter sequence encodes a heat stable antigen comprising a heterologous detectable domain in or in place of all or part of an extracellular domain sequence. In one aspect
30 (optionally), the all or a portion of the extracellular domain is deleted and the heterologous detectable domain is the same size or substantially the same size as the deleted sequence. In one aspect (optionally), the heat stable antigen is a murine heat stable antigen (CD24, see below) or a human heat stable antigen.

In one aspect of any of the methods of the invention, the level of reporter sequence expression is assessed or determined by RNA message or protein expression detection methods. The reporter sequence can be expressed on the extracellular surface of the host cell (e.g., within or in place of an HSA extracellular domain), thus, in one aspect, the level of reporter sequence expression is determined by flow cytometric analysis, and in one aspect (optionally), the flow cytometric analysis is by Fluorescence Activated Cell Sorter (FACS). The level of reporter sequence expression can be determined by molecular detection methods, e.g., polymerase chain reaction (PCR), wherein in one aspect (optionally) the PCR comprises real-time PCR (e.g., 7300 or 7500 REAL-TIME™ PCR Systems, Applied Biosystems, Foster City, CA).

In one aspect of any of the methods of the invention, the recombinant virus comprises an RNA virus or a DNA virus, e.g., a retrovirus, a hepadnavirus, a flavivirus, a herpesvirus (e.g., a herpes simplex virus), an Epstein-Barr virus, an adenovirus, an adeno-associated virus, a papilloma virus, a vaccinia virus, and the like. In one aspect, the recombinant virus comprises a virus from the family *Alphaviridae*, *Flaviviridae*, *Hepadnaviridae*, *Papovaviridae*, *Parvoviridae*, *Herpesviridae*, *Poxviridae*, *Paramyxoviridae*, *Rhabdoviridae*, *Retroviridae*, e.g., onco-retroviruses, the Spuma-retroviruses and the Lenti-retroviruses. The virus can be a human immunodeficiency virus (HIV), e.g., a human immunodeficiency virus-1 (HIV-1) or HIV-2.

In one aspect of any of the methods of the invention, the recombinant viruses are introduced into the host cells *in vivo* or *in vitro*, e.g., by infection, transfection, transduction, lipofection, electroporation, injection (e.g., by jet gun) or any other means. A recombinant virus can be introduced into the host cells *in vitro*, and the host cells are subsequently implanted or transferred *in vivo* to a non-human animal, and optionally the non-human animal is a mouse, a rat or a non-human primate. In one aspect of any of the methods of the invention, the recombinant viruses are introduced into the host cells *in vivo*. In one aspect, the recombinant viruses are introduced *in vivo* into host cells in a non-human animal, e.g., where the non-human animal is a mouse, a rat or a non-human primate, e.g., a SCID-Hu mice or a chimpanzee.

The invention provides host cells, e.g., mammalian cells, comprising a recombinant virus of the invention, a chimeric nucleic acid construct of the invention, or a chimeric polypeptide of the invention. The invention provides transgenic non-human animals, e.g., a non-human mammal, comprising a recombinant virus of the invention, a chimeric nucleic acid construct of the invention, or a chimeric polypeptide of the invention.

The invention provides kits comprising a host cell of the invention, e.g., a mammalian cell, a recombinant virus of the invention, a chimeric nucleic acid construct of the invention, or a chimeric polypeptide of the invention. In one aspect (optionally), the kit comprises instructions to practice a method of the invention.

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The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims. All publications, patents, patent applications and ATCC or UniProtKB/Swiss-Prot deposits, cited
10 herein are hereby expressly incorporated by reference for all purposes.

BRIEF DESCRIPTION OF DRAWINGS

The following drawings are illustrative of aspects of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figure 1 illustrates an exemplary reporter construct of the invention (SEQ ID
15 NO:1) (bottom sequence), VPR-HSA-HA, in the *vpr* locus of HIV-1; and, a VPR (SEQ ID NO:4) (top sequence) and VPR-HSA (SEQ ID NO:5) (middle sequence) construct, also in the *vpr* locus of HIV-1, as discussed in detail in Example 1, below.

Figure 2 illustrates data showing detection of HIV-1-infected cells by fluorescent antibody surface staining for the reporter proteins or intracellular Gag, as discussed in detail in
20 Example 1, below.

Figure 3 illustrates data showing the specific detection of cells infected by the HIV-1-HSA reporter construct or the exemplary HIV-1-HSA-HA construct of the invention by fluorescent antibody surface staining, as discussed in detail in Example 1, below.

Figure 4 illustrates an exemplary real time PCR strategy for specific detection of
25 HIV-1 tagged with HSA and HSA-HA reporters, as discussed in detail in Example 1, below.

Figure 5 is an illustration of a graph summarizing data showing the growth of HIV-1 with the HIV-1-HSA reporter construct or the exemplary HIV-1-HSA-HA construct of the invention in PBMC, as discussed in detail in Example 1, below.

Figure 6 graphically illustrates data showing the relative growth of HIV-1
30 containing wild type or mutated Nef in the absence or presence of HIV-1-specific CTL, as discussed in detail in Example 1, below.

Figure 7 graphically illustrates data showing the growth of two viruses in mixed cultures over time, as quantitated by real time PCR for each virus, using an exemplary method and exemplary recombinant viruses of the invention, as discussed in detail in Example 1, below.

Figure 8 graphically illustrates data showing results from the screening of 11 different clones of HIV for the fitness impact of point mutations in the Gag protein using an exemplary method and exemplary recombinant viruses of the invention, as discussed in detail in Example 1, below.

Like reference symbols in the various drawings indicate like elements.

DETAILED DESCRIPTION

The invention provides novel compositions comprising reporter constructs comprising heat stable antigen (HSA) reporter and heterologous (non-HSA) marker sequences, e.g., to determine relative viral fitness, and methods for making and using them. The invention also provides viruses, plasmids, phages, artificial chromosomes or any vector comprising a reporter construct of the invention. Also provided are kits comprising a reporter construct, a chimeric nucleic acid, a host cell, a chimeric polypeptide and/or a recombinant virus of the invention, including in one aspect instructions for practicing the methods provided herein.

In one aspect, the invention provides reporters constructs comprising heat stable antigen (HSA) reporter sequences comprising detectable heterologous (non-HSA) marker sequences that can be used in viral detection assays and viral growth assays, and methods for making and using same. In one aspect, the invention provides methods for determining the relative fitness of at least two viruses, e.g., under a selective constraint or a particular growth condition, including determining differences in growth rate of the different viruses under the constraints of an immune response, a drug, a vaccine or a particular set of environmental conditions. Thus, the invention provides compositions and methods to determine the relative viral fitness (i.e., the ability of a given virus to replicate) of two or more viruses. Likewise, the invention provides compositions and methods (e.g., viral competition assays) able to measure the relative fitness of at least two viruses over the entire life cycle of the virus(es).

The invention can be practiced *in vivo* or *in vitro*, or, a combination of both. In one aspect, the methods of the invention allow specific detection of at least two viruses within a mixture – which can be an *in vitro* system (e.g., designed have exact environmental parameters, e.g., to simulate the intracellular conditions of any particular cell), or an infected or a transduced cell, or tissue or organ, and to be able to quantitate each virus individually within the one *in vitro*

system, or *in vivo* system, including being able to quantitate each virus individually within the one cell, one culture, one tissue or one organ.

In one aspect, the invention provides reporter nucleic acid constructs based on the small murine heat stable antigen (HSA) protein, which is expressed on the surface of cell, including virally infected cells. In this exemplary aspect, an HSA reporter is inserted in the *vpr* reading frame of HIV, leaving *nef* intact. Nine amino acids from the extracellular domain of HSA are replaced with an influenza hemagglutinin (HA) antibody epitope (HSA-HA) (note: in other aspects, additional residues or all of the extracellular domain of HSA is replaced with a “detectable” sequence, which can include either a “detectable” nucleic acid, e.g., by PCR, or a “detectable” protein, e.g., an epitope encoded by an antibody). In aspect, the “detectable domain” of the reporter construct is expressed on the surface of the infected cell. In another aspect, the heterologous (to the virus) nucleic acid inserted into the deleted section of the viral genome (e.g., all or some of the *vpr* reading frame) is detected by molecular detection techniques such as PCR.

In one aspect, in practicing the methods of the invention, at least two different viruses (which can be different by only one (or more) nucleic acid or amino acid residues, or, be completely different viruses; e.g., the at least two different viruses can be members of different orders, families, genera, species, strains, substrains or clades of a virus, such as HIV, or they can be members of the same strain, substrain or clade of a virus but differ in sequence or viral protein or nucleic acid expression) are used to infect or transduce a host cell. Each different virus comprises its own detectable moiety, e.g., an epitope (such as hemagglutinin (HA)) expressed in the extracellular domain, e.g., an extracellular domain encoded by a construct of the invention comprising a chimeric HSA. Thus, in one aspect of the methods of the invention, antibodies for epitope, e.g., HSA, HA and the like, specifically detect each different reporter construct – effectively also reporting the relative fitness of each two different mutant, strain, substrain or clade of virus, such as HIV, used in this growth competition assay.

In one aspect, each different reporter construct (i.e., each different recombinant virus used in the growth competition assay) is measured by a molecular detection protocol, e.g., by PCR, including real-time PCR quantitation – thus allowing a “real-time” monitoring of the relative fitness of each of the different orders, families, genera, species, mutants, strains, substrains or clades of virus used in the growth competition assay of the invention. The growth of viruses “tagged” with each reporter allows precise assessment of the relative growth of different viruses, e.g., viruses differing in mutations of interest. Thus, the invention provides for

“paired” or multiple reporter constructs (e.g., recombinant viruses) each having a distinct “detectable” moiety to offer a useful standardized method for measurement of viral fitness (e.g., HIV-1 fitness) in competition assays.

The invention provides reporter constructs for HIV-1, which in one aspect can be used for identifying infected cells and studying viral replication. Using the viral constructs of the invention (containing a different “reporter” sequence for each virus, virus mutant or variation to be studied, or compared in the growth assay) enables the study of effects of infection *in vitro* and *in vivo*, allowing characterization of the viruses and delineation of infected cells. In one aspect, the compositions and methods of the invention can be used in conjunction with tagging infected cells through direct fluorescence, e.g. green fluorescent protein, GFP, or tag infected cells by reporter-specific fluorescent staining on the surface of infected cells, using, e.g., placental alkaline phosphatase, PLAP) (see, e.g., Chen (1996) J. Virol. 70:6044-6053).

However, in one aspect, unlike known reporter genes used for detecting HIV-1 or HIV growth, the instant invention provides nucleic acid constructs that are not artificially large in size (e.g., they do not incorporate large reporter genes such as GFP and PLAP, which are hundreds of amino acids in size), and thus do not create artifactual data or data skewed by an unnaturally large sized viral genome. Additionally, because the nucleic acid constructs of the invention do not interrupt, modify or delete any viral gene or protein necessary or influential on “viral fitness” (e.g., *nef* or *pol*) (noting, e.g., Nef is important for viral growth in primary CD4⁺ T lymphocytes, e.g., see Spina (1994) J. Exp. Med. 179:115-123), results from studies using nucleic acid constructs of the invention do not create artifactual data or data skewed by a modified viral genome. The constructs of the instant invention do not interrupt, modify or delete any viral gene or protein that play(s) a crucial role(s) in the immunopathogenesis of HIV-1 infection.

Because the nucleic acid (gene) constructs of the invention do not comprise artificially large inserts, they avoid the potential genetic instability problem associated with large reporter genes, particularly in models of HIV-1 replication *in vivo* (Jamieson (1998) J. Virol. 72:6520-6526). Additionally, because the nucleic acid constructs of the invention do not comprise artificially large inserts, they avoid the potential problem of losing the insert over multiple rounds of viral replication (because a large insert is a large target for the rapid mutation rate of HIV-1, its expression can be lost rapidly over multiple rounds of viral replication). Thus, in one aspect, the invention uses very small reporter sequences inserted into HIV-1, e.g., murine CD24, or heat stable antigen (HSA) (see, e.g., Jamieson (1998) *supra*) or equivalent proteins.

In one aspect, the invention provides methods for measuring replication fitness which can be adapted to viruses, including, but not limited to human immunodeficiency virus (HIV), hepadnaviruses (human hepatitis B virus), flaviviruses (human hepatitis C virus) and herpesviruses (human cytomegalovirus) (also including: see additional list, above). The structure, life cycle and genetic elements of the viruses which comprise the recombinant viruses of the invention, and can be used in the methods of the invention, e.g., which can be tested in the drug susceptibility and resistance tests of this invention, are well known to one of ordinary skill in the art.

Thus, the invention provides compositions and methods to assess the antiviral effect of an immune response, e.g., a CTL response, e.g., a cytotoxic CD8⁺ T cell response. The compositions and methods allow analysis of the complex interplay of multiple virologic and cellular factors in one assay. Accordingly, in one aspect, CTL assays of the invention provide a relatively complete reflection of the efficiency of an immune response, e.g., CTL or vaccine efficiency.

Generating and Manipulating Nucleic Acids

The invention provides chimeric nucleic acid constructs comprising a nucleotide sequence encoding a heat stable antigen (HSA) reporter sequence comprising a detectable heterologous (non-HSA) marker sequence, and recombinant viruses comprising these chimeric nucleic acid constructs. The invention also provides expression cassettes, e.g., vectors such as expression vectors, comprising chimeric nucleic acid constructs of the invention, which include polynucleotides which encode the polypeptides of the invention (e.g., the chimeric HSA-extracellular epitope polypeptides of the invention).

The nucleic acids of the invention (e.g., chimeric nucleic acid constructs of the invention) can be made, isolated and/or manipulated by, e.g., cloning and expression of cDNA libraries, amplification of message or genomic DNA by PCR, and the like. In practicing the methods of the invention, nucleic acids of the invention can be modified by manipulating a template nucleic acid, as described herein. The invention can be practiced in conjunction with any method or protocol or device known in the art, which are well described in the scientific and patent literature.

The phrases "nucleic acid" or "nucleic acid sequence" as used herein can comprise reference to an oligonucleotide, nucleotide, polynucleotide, or to a fragment of any of these, to DNA or RNA of genomic or synthetic origin which may be single-stranded or double-stranded and may represent a sense or antisense (complementary) strand, to peptide nucleic acid

(PNA), or to any DNA-like or RNA-like material, natural or synthetic in origin. The phrases "nucleic acid" or "nucleic acid sequence" includes oligonucleotide, nucleotide, polynucleotide, or to a fragment of any of these, to DNA or RNA (e.g., mRNA, rRNA, tRNA, iRNA) of genomic or synthetic origin which may be single-stranded or double-stranded and may represent
5 a sense or antisense strand, to peptide nucleic acid (PNA), or to any DNA-like or RNA-like material, natural or synthetic in origin, including, e.g., iRNA, ribonucleoproteins (e.g., e.g., double stranded iRNAs, e.g., iRNPs). The term encompasses nucleic acids, i.e., oligonucleotides, containing known analogues of natural nucleotides. The term also encompasses nucleic-acid-like structures with synthetic backbones, see e.g., Mata (1997) Toxicol. Appl. Pharmacol. 144:189-197; Strauss-Soukup (1997) Biochemistry 36:8692-8698; Samstag (1996) Antisense Nucleic Acid Drug Dev 6:153-156. "Oligonucleotide" includes either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized.

The term "gene", e.g., as in a *nef* or a *vpr* gene, means the segment of DNA
15 involved in producing a polypeptide chain; and can include regions preceding and following the coding region (leader and trailer) as well as, where applicable, intervening sequences (introns) between individual coding segments (exons). "Operably linked" as used herein refers to a functional relationship between two or more nucleic acid (e.g., DNA) segments. In one aspect, it refers to the functional relationship of transcriptional regulatory sequence to a transcribed
20 sequence. For example, a promoter is operably linked to a coding sequence, such as a nucleic acid of the invention, if it stimulates or modulates the transcription of the coding sequence in an appropriate host cell or other expression system. In one aspect, promoter transcriptional regulatory sequences that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, i.e., they are cis-acting. However, some transcriptional
25 regulatory sequences, such as enhancers, need not be physically contiguous or located in close proximity to the coding sequences whose transcription they enhance.

In one aspect, a nucleic acid construct of the invention comprises an "expression cassette" or a vector, e.g., an "expression vector"; the term as used herein can refer to a nucleotide sequence which is capable of affecting expression of a structural gene (e.g., a protein
30 coding sequence, such a chimeric HSA-HA (extracellular domain) chimeric protein of the invention) in a host compatible with such sequences. Expression cassettes include at least a promoter operably linked with the polypeptide coding sequence; and, optionally, with other sequences, e.g., transcription termination signals. Additional factors necessary or helpful in

effecting expression may also be used, e.g., enhancers. Thus, expression cassettes also include plasmids, expression vectors, recombinant viruses, any form of recombinant "naked DNA" vector, and the like. A "vector" comprises a nucleic acid which can infect, transfect, transiently or permanently transduce a cell. It will be recognized that a vector can be a naked nucleic acid, or a nucleic acid complexed with protein or lipid. The vector optionally comprises viral or bacterial nucleic acids and/or proteins, and/or membranes (e.g., a cell membrane, a viral lipid envelope, etc.). Vectors include, but are not limited to replicons (e.g., RNA replicons, bacteriophages) to which fragments of DNA may be attached and become replicated. Vectors thus include, but are not limited to RNA, autonomous self-replicating circular or linear DNA or RNA (e.g., plasmids, viruses, and the like), and include both the expression and non-expression plasmids. Where a recombinant microorganism, host cell or cell culture is described as hosting a nucleic acid of the invention, e.g., an expression vector, this includes both extra-chromosomal circular and linear DNA and DNA that has been incorporated into the host chromosome(s). Where a recombinant virus or vector of the invention is being maintained by a host cell, the vector or recombinant virus may either be stably replicated by the cells during mitosis as an autonomous structure, or is incorporated within the host's genome.

The skilled artisan will recognize that any compound used in the methods of the invention (e.g., catalytic, starting or intermediate compounds, e.g., for drugs to be tested in the methods of the invention) can be synthesized using a variety of procedures and methodologies, which are well described in the scientific and patent literature., e.g., Organic Syntheses Collective Volumes, Gilman *et al.* (Eds) John Wiley & Sons, Inc., NY. The invention can be practiced in conjunction with any method or protocol known in the art, which are well described in the scientific and patent literature.

The nucleic acids used to practice this invention, whether RNA, iRNA, antisense nucleic acid, cDNA, genomic DNA, vectors, viruses or hybrids thereof, may be isolated from a variety of sources, genetically engineered, amplified, and/or expressed/ generated recombinantly. Recombinant polypeptides (e.g., chimeric HSAs of the invention) generated from these nucleic acids can be individually isolated or cloned and tested for a desired activity. Any recombinant expression system can be used, including bacterial, mammalian, yeast, insect or plant cell expression systems.

Alternatively, these nucleic acids can be synthesized *in vitro* by well-known chemical synthesis techniques, as described in, e.g., Adams (1983) J. Am. Chem. Soc. 105:661; Belousov (1997) Nucleic Acids Res. 25:3440-3444; Frenkel (1995) Free Radic. Biol. Med.

19:373-380; Blommers (1994) Biochemistry 33:7886-7896; Narang (1979) Meth. Enzymol. 68:90; Brown (1979) Meth. Enzymol. 68:109; Beaucage (1981) Tetra. Lett. 22:1859; U.S. Patent No. 4,458,066.

Techniques for the manipulation of nucleic acids, such as, e.g., subcloning,
5 labeling probes (e.g., random-primer labeling using Klenow polymerase, nick translation, amplification), sequencing, hybridization and the like are well described in the scientific and patent literature, see, e.g., Sambrook, ed., MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York
10 (1997); LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993).

The nucleic acids of the invention, e.g., the chimeric nucleic acid constructs of the invention, can be contained in any vehicle, e.g., mammalian artificial chromosomes (MACs),
15 see, e.g., U.S. Patent Nos. 5,721,118; 6,025,155; human artificial chromosomes, see, e.g., Rosenfeld (1997) Nat. Genet. 15:333-335; yeast artificial chromosomes (YAC); bacterial artificial chromosomes (BAC); P1 artificial chromosomes, see, e.g., Woon (1998) Genomics 50:306-316; P1-derived vectors (PACs), see, e.g., Kern (1997) Biotechniques 23:120-124; cosmid, recombinant viruses, phages or plasmids.

Recombinant viruses of the invention can comprise any virus, e.g., as described herein, e.g., a recombinant RNA or DNA virus, e.g., a retrovirus, a hepadnavirus, a flavivirus, a herpesvirus (e.g., a herpes simplex virus), an Epstein-Barr virus, an adenovirus, an adeno-associated virus, a papilloma virus, a vaccinia virus, and the like. In one aspect, exemplary recombinant viruses of the invention (as described herein, see Example 1, below), used the HIV
25 genome NL4-3 as the parental virus sequence. The original description of NL4-3 (the parental virus) sequence is Adachi (1986) Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol 59:284-291. The plasmid constructs containing NL4-3 in two separate pieces as used to construct exemplary recombinant viruses of the invention are described in, e.g., Gibbs
30 (1994) Construction and *in vitro* properties of HIV-1 mutants with deletions in "nonessential" genes. AIDS Res. Hum. Retroviruses 10:343-350.

Transcriptional and translational control sequences

The invention provides nucleic acids (e.g., chimeric nucleic acid constructs) operatively linked to expression (e.g., transcriptional or translational) control sequence(s), e.g., promoters or enhancers, to direct or modulate RNA synthesis/ expression. The expression control sequence can be in a recombinant virus, an expression vector and the like. In addition to viral promoters, the chimeric nucleic acids of the invention can be operatively linked to any transcriptional or translational control sequence. Exemplary bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda PR, PL and trp. Exemplary eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, long terminal repeats (LTRs) from retroviruses (e.g., HIV LTR), and mouse metallothionein I. The control sequences can be constitutive or inducible.

Host cells and transformed cells

The invention also provides a transformed, transduced or infected cell comprising a chimeric nucleic acid sequence of the invention, e.g., a chimeric nucleic acid construct and/or a recombinant virus of the invention, and/or a chimeric polypeptide of the invention. The invention provides methods for determining the relative fitness of at least two viruses, comprising introducing a first recombinant virus comprising a first reporter sequence into a host cell, and introducing at least a second recombinant virus comprising at least a second reporter sequence into the host cell, wherein each reporter sequence is distinguishably detectable from the other reporter sequences (wherein the host cell is permissive for growth of the virus); and culturing the host cell. Any host cell, or *in vitro* simulation of a host cell, can be used, e.g., a mammalian host cell system.

Suitable host cells to practice the methods of the invention can be derived from tissues, including human tissues, and/or cells; and in one aspect, tissues and cells which are the principle targets of viral infection are used as host cells. For example, for HIV studies these include human cells such as human glial cells, T cells, monocytes, macrophage, dendritic cells, Langerhans cells, hematopoietic stem cells, stem cells or precursor cells, and other cells. In the case of HBV, suitable host cells include hepatoma cell lines (HepG2, Huh 7), primary human hepatocytes, mammalian cells which can be infected by pseudotyped virus, and other cells. Human derived host cells will assure that the anti-viral drug will enter the cell efficiently and be converted by the cellular enzymatic machinery into the metabolically relevant form of the anti-viral inhibitor.

Host cells that can be used to practice the invention, particularly for using HIV-based recombinant viruses of the invention, include 293 human embryonic kidney cells (293, Graham (1977) J. Gen Virol. 36:59), BOSC23 (Pear (1993) Proc. Natl. Acad. Sci. USA 90:8392), tsa54 and tsa201 cell lines (Heinzel (1988) J. Virol. 62:3738); for HBV HepG2 (Galle
5 (1990) Drug Res. 40:1380-1382). Other alternative host cells that can be used to practice the invention include human T cell leukemia cell lines including Jurkat (ATCC T1B-152), H9 (ATCC HTB-176), CEM (ATCC CCL-119), HUT78 (ATCC T1B-161), and derivatives thereof.

A nucleic acid used to practice the invention (e.g., a chimeric nucleic acid construct and/or a recombinant virus of the invention) can be introduced into the host cells using
10 any of a variety of techniques, including transformation, transfection, transduction, viral infection, gene guns, or Ti-mediated gene transfer. Particular methods include calcium phosphate transfection, DEAE-Dextran mediated transfection, lipofection, or electroporation. Other exemplary methods include CaPO₄ precipitation, liposome fusion, lipofection (e.g., LIPOFECTIN™), electroporation, viral infection, etc. The nucleic acids used to practice the
15 invention may stably integrate into the genome of the host cell (for example, with retroviral introduction) or may exist either transiently or stably in the cytoplasm (i.e. through the use of traditional plasmids, utilizing standard regulatory sequences, selection markers, etc.). Retroviral vectors capable of transfecting host cell targets can be used.

Where appropriate, the engineered host cells can be cultured in conventional
20 nutrient media modified as appropriate for activating promoters, selecting transformants or expressing the chimeric polypeptides of the invention. Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter may be induced by appropriate means (e.g., temperature shift or chemical induction) and the cells may be cultured for an additional period to allow them to produce the desired polypeptide (e.g.,
25 a chimeric HSA) or fragment thereof.

Cell-free translation systems can also be employed to practice the methods of the invention or to produce a chimeric polypeptide of the invention. Cell-free translation systems can use mRNAs transcribed from a DNA construct comprising a promoter operably linked to a nucleic acid encoding the polypeptide or fragment thereof. In some aspects, the DNA construct
30 may be linearized prior to conducting an *in vitro* transcription reaction. The transcribed mRNA is then incubated with an appropriate cell-free translation extract, such as a rabbit reticulocyte extract, to produce the desired polypeptide or fragment thereof.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means and the resulting crude extract is retained for further purification. Microbial cells employed for expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. Such methods are well known to those skilled in the art. An expressed polypeptide or fragment thereof can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the polypeptide. High performance liquid chromatography (HPLC) can be employed for final purification steps.

Amplification of Nucleic Acids

In practicing the methods of the invention the level of reporter sequence expression can be assessed or determined by RNA message or protein expression detection methods. The level of reporter sequence expression can be determined by molecular detection methods, e.g., polymerase chain reaction (PCR), wherein in one aspect (optionally) the PCR comprises real-time PCR (e.g., 7300 or 7500 REAL-TIME™ PCR Systems, Applied Biosystems, Foster City, CA).

In practicing the invention, nucleic acids of the invention and nucleic acids encoding enzymes of the invention, or modified nucleic acids of the invention, also can be reproduced by amplification. Amplification can also be used to clone or modify the nucleic acids of the invention. Thus, the invention provides amplification primer sequence pairs for amplifying nucleic acids of the invention, particular sequence pairs designed to amplify only one of two or more viruses used in the viral fitness ("growth competition") assays of the invention. One of skill in the art can design amplification primer sequence pairs for any part of or the full length of these sequences.

The skilled artisan can select and design suitable oligonucleotide amplification primers. Amplification methods are also well known in the art, and include, e.g., polymerase chain reaction, PCR (see, e.g., PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS, ed. Innis, Academic Press, N.Y. (1990) and PCR STRATEGIES (1995), ed. Innis, Academic Press, Inc., N.Y., ligase chain reaction (LCR) (see, e.g., Wu (1989) Genomics 4:560; Landegren (1988) Science 241:1077; Barringer (1990) Gene 89:117); transcription amplification (see, e.g., Kwoh (1989) Proc. Natl. Acad. Sci. USA 86:1173); and, self-sustained

sequence replication (see, e.g., Guatelli (1990) Proc. Natl. Acad. Sci. USA 87:1874); Q Beta replicase amplification (see, e.g., Smith (1997) J. Clin. Microbiol. 35:1477-1491), automated Q-beta replicase amplification assay (see, e.g., Burg (1996) Mol. Cell. Probes 10:257-271) and other RNA polymerase mediated techniques (e.g., NASBA, Cangene, Mississauga, Ontario); see also Berger (1987) Methods Enzymol. 152:307-316; Sambrook; Ausubel; U.S. Patent Nos. 4,683,195 and 4,683,202; Sooknanan (1995) Biotechnology 13:563-564.

As used herein, "replication capacity" is defined herein is a measure of how well the virus replicates. This may also be referred to as viral fitness. In one embodiment, replication capacity can be measured by evaluating the ability of the virus to replicate in a single round of replication.

Transgenic non-human animals

The invention provides transgenic non-human animals comprising a chimeric nucleic acid, a chimeric polypeptide, an expression cassette or vector, a recombinant virus, or an infected, transduced, transfected or transformed cell of the invention. The invention also provides methods of making and using these transgenic non-human animals, e.g., in the methods of this invention.

The transgenic non-human animals can be, e.g., dogs, goats, rabbits, sheep, pigs, cows, rats and mice, comprising the nucleic acids of the invention. These animals can be used, e.g., to practice *in vivo* viral fitness assays of the invention. The coding sequences for the polypeptides to be expressed in the transgenic non-human animals can be designed to be constitutive, or, under the control of tissue-specific, developmental-specific or inducible transcriptional regulatory factors. Transgenic non-human animals can be designed and generated using any method known in the art; see, e.g., U.S. Patent Nos. 6,211,428; 6,187,992; 6,156,952; 6,118,044; 6,111,166; 6,107,541; 5,959,171; 5,922,854; 5,892,070; 5,880,327; 5,891,698; 5,639,940; 5,573,933; 5,387,742; 5,087,571, describing making and using transformed cells and eggs and transgenic mice, rats, rabbits, sheep, pigs and cows. See also, e.g., Pollock (1999) J. Immunol. Methods 231:147-157, describing the production of recombinant proteins in the milk of transgenic dairy animals; Baguisi (1999) Nat. Biotechnol. 17:456-461, demonstrating the production of transgenic goats. U.S. Patent No. 6,211,428, describes making and using transgenic non-human mammals which express in their brains a nucleic acid construct comprising a DNA sequence. U.S. Patent No. 5,387,742, describes injecting cloned recombinant or synthetic DNA sequences into fertilized mouse eggs, implanting the injected eggs in pseudo-pregnant females, and growing to term transgenic mice whose cells

express proteins related to the pathology of Alzheimer's disease. U.S. Patent No. 6,187,992, describes making and using a transgenic mouse whose genome comprises a disruption of the gene encoding amyloid precursor protein (APP).

Polypeptides and peptides

5 In one aspect, the invention provides isolated, synthetic or recombinant polypeptides comprising a chimeric heat stable antigen (HSA) reporter sequence. In one aspect, the invention provides polypeptides encoded by a chimeric nucleic acid comprising a sequence as set forth in SEQ ID NO:1 (VPR-HSA-HA) or SEQ ID NO:21 (Nef-HSA-HA).

10 In one aspect, the heat stable antigen (HSA) reporter sequence used in the chimeric constructs, and used to practice the methods of the invention, comprise a murine heat stable antigen (HSA) surface protein, also called CD24 (synonyms have been "M1/69-J11D heat stable antigen", nectadrin, LY-52, X62 heat stable antigen, and R13-AG) see, e.g., UniProtKB/Swiss-Prot entry (primary accession number) P24807; P26691; Kay (1990) J. Immunol. 145(6):1952-1959. Human or other mammalian (e.g., rat, chimpanzee, etc.) heat
15 stable antigen (HSA) sequences can also be used.

Polypeptides and peptides of the invention can be isolated from natural sources, be synthetic, or be recombinantly generated polypeptides. Peptides and proteins can be recombinantly expressed *in vitro* or *in vivo*. The peptides and polypeptides of the invention can be made and isolated using any method known in the art. Polypeptide and peptides of the
20 invention can also be synthesized, whole or in part, using chemical methods well known in the art. See e.g., Caruthers (1980) Nucleic Acids Res. Symp. Ser. 215-223; Horn (1980) Nucleic Acids Res. Symp. Ser. 225-232; Banga, A.K., Therapeutic Peptides and Proteins, Formulation, Processing and Delivery Systems (1995) Technomic Publishing Co., Lancaster, PA. For example, peptide synthesis can be performed using various solid-phase techniques (see e.g.,
25 Roberge (1995) Science 269:202; Merrifield (1997) Methods Enzymol. 289:3-13) and automated synthesis may be achieved, e.g., using the ABI 431A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer.

The peptides and polypeptides of the invention, as defined above, include all "mimetic" and "peptidomimetic" forms. The terms "mimetic" and "peptidomimetic" refer to a
30 synthetic chemical compound which has substantially the same structural and/or functional characteristics of the polypeptides of the invention. The mimetic can be either entirely composed of synthetic, non-natural analogues of amino acids, or, is a chimeric molecule of partly natural peptide amino acids and partly non-natural analogs of amino acids. The mimetic

can also incorporate any amount of natural amino acid conservative substitutions as long as such substitutions also do not substantially alter the mimetic's structure and/or activity.

Polypeptide mimetic compositions of the invention can contain any combination of non-natural structural components. In alternative aspect, mimetic compositions of the invention include one or all of the following three structural groups: a) residue linkage groups other than the natural amide bond ("peptide bond") linkages; b) non-natural residues in place of naturally occurring amino acid residues; or c) residues which induce secondary structural mimicry, i.e., to induce or stabilize a secondary structure, e.g., a beta turn, gamma turn, beta sheet, alpha helix conformation, and the like. For example, a polypeptide of the invention can be characterized as a mimetic when all or some of its residues are joined by chemical means other than natural peptide bonds. Individual peptidomimetic residues can be joined by peptide bonds, other chemical bonds or coupling means, such as, e.g., glutaraldehyde, N-hydroxysuccinimide esters, bifunctional maleimides, N,N'-dicyclohexylcarbodiimide (DCC) or N,N'-diisopropylcarbodiimide (DIC). Linking groups that can be an alternative to the traditional amide bond ("peptide bond") linkages include, e.g., ketomethylene (e.g., -C(=O)-CH₂- for -C(=O)-NH-), aminomethylene (CH₂-NH), ethylene, olefin (CH=CH), ether (CH₂-O), thioether (CH₂-S), tetrazole (CN₄-), thiazole, retroamide, thioamide, or ester (see, e.g., Spatola (1983) in *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*, Vol. 7, pp 267-357, "Peptide Backbone Modifications," Marcell Dekker, NY).

A polypeptide of the invention can also be characterized as a mimetic by containing all or some non-natural residues in place of naturally occurring amino acid residues. Non-natural residues are well described in the scientific and patent literature; a few exemplary non-natural compositions useful as mimetics of natural amino acid residues and guidelines are described below. Mimetics of aromatic amino acids can be generated by replacing by, e.g., D- or L- naphylalanine; D- or L- phenylglycine; D- or L-2 thieneylalanine; D- or L-1, -2, 3-, or 4-pyreneylalanine; D- or L-3 thieneylalanine; D- or L-(2-pyridinyl)-alanine; D- or L-(3-pyridinyl)-alanine; D- or L-(2-pyrazinyl)-alanine; D- or L-(4-isopropyl)-phenylglycine; D-(trifluoromethyl)-phenylglycine; D-(trifluoromethyl)-phenylalanine; D-p-fluoro-phenylalanine; D- or L-p-biphenylphenylalanine; D- or L-p-methoxy-biphenylphenylalanine; D- or L-2-indole(alkyl)alanines; and, D- or L-alkylainines, where alkyl can be substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, isopropyl, iso-butyl, sec-isotyl, iso-pentyl, or a non-acidic amino acids. Aromatic rings of a non-natural amino acid include, e.g., thiazolyl, thiophenyl, pyrazolyl, benzimidazolyl, naphthyl, furanyl, pyrrolyl, and pyridyl aromatic rings.

Mimetics of acidic amino acids can be generated by substitution by, e.g., non-carboxylate amino acids while maintaining a negative charge; (phosphono)alanine; sulfated threonine. Carboxyl side groups (e.g., aspartyl or glutamyl) can also be selectively modified by reaction with carbodiimides ($R'-N-C-N-R'$) such as, e.g., 1-cyclohexyl-3(2-morpholinyl)-(4-ethyl) carbodiimide or 1-ethyl-3(4-azonia-4,4-dimethylpentyl) carbodiimide. Aspartyl or glutamyl can also be converted to asparaginy and glutaminy residues by reaction with ammonium ions. Mimetics of basic amino acids can be generated by substitution with, e.g., (in addition to lysine and arginine) the amino acids ornithine, citrulline, or (guanidino)-acetic acid, or (guanidino)alkyl-acetic acid, where alkyl is defined above. Nitrile derivative (e.g., containing the CN-moiety in place of COOH) can be substituted for asparagine or glutamine. Asparaginy and glutaminy residues can be deaminated to the corresponding aspartyl or glutamyl residues. Arginine residue mimetics can be generated by reacting arginy with, e.g., one or more conventional reagents, including, e.g., phenylglyoxal, 2,3-butanedione, 1,2-cyclo-hexanedione, or ninhydrin, in one aspect under alkaline conditions. Tyrosine residue mimetics can be generated by reacting tyrosyl with, e.g., aromatic diazonium compounds or tetranitromethane. N-acetylimidizol and tetranitromethane can be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Cysteine residue mimetics can be generated by reacting cysteiny residues with, e.g., alpha-haloacetates such as 2-chloroacetic acid or chloroacetamide and corresponding amines; to give carboxymethyl or carboxyamidomethyl derivatives. Cysteine residue mimetics can also be generated by reacting cysteiny residues with, e.g., bromo-trifluoroacetone, alpha-bromo-beta-(5-imidozoyl) propionic acid; chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide; methyl 2-pyridyl disulfide; p-chloromercuribenzoate; 2-chloromercuri-4 nitrophenol; or, chloro-7-nitrobenzo-oxa-1,3-diazole. Lysine mimetics can be generated (and amino terminal residues can be altered) by reacting lysiny with, e.g., succinic or other carboxylic acid anhydrides. Lysine and other alpha-amino-containing residue mimetics can also be generated by reaction with imidoesters, such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitro-benzenesulfonic acid, O-methylisourea, 2,4, pentanedione, and transamidase-catalyzed reactions with glyoxylate. Mimetics of methionine can be generated by reaction with, e.g., methionine sulfoxide. Mimetics of proline include, e.g., pipecolic acid, thiazolidine carboxylic acid, 3- or 4- hydroxy proline, dehydropyrolidine, 3- or 4-methylproline, or 3,3,-dimethylproline. Histidine residue mimetics can be generated by reacting histidyl with, e.g., diethylprocarbonate or para-bromophenacyl bromide. Other mimetics include, e.g., those generated by hydroxylation of proline and lysine;

phosphorylation of the hydroxyl groups of seryl or threonyl residues; methylation of the alpha-amino groups of lysine, arginine and histidine; acetylation of the N-terminal amine; methylation of main chain amide residues or substitution with N-methyl amino acids; or amidation of C-terminal carboxyl groups.

5 Antibodies and Antibody-based screening methods

The invention provides invention provides chimeric nucleic acid constructs comprising a nucleotide sequence encoding a heat stable antigen (HSA) reporter sequence comprising a detectable heterologous (non-HSA) marker sequence; and in one aspect, the detectable heterologous sequence can encode an epitope recognized by an antibody, including
 10 e.g., single stranded antibodies, CDRs, antigen binding sites and the like. The term "antibody" can include a peptide or polypeptide derived from, modeled after or substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, capable of specifically binding an antigen or epitope, see, e.g. Fundamental Immunology, Third Edition, W.E. Paul, ed., Raven Press, N.Y. (1993); Wilson (1994) J. Immunol. Methods 175:267-273; Yarmush (1992)
 15 J. Biochem. Biophys. Methods 25:85-97. The term antibody includes antigen-binding portions, i.e., "antigen binding sites," (e.g., fragments, subsequences, complementarity determining regions (CDRs)) that retain capacity to bind antigen, including (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a
 20 Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Single chain antibodies are also included by reference in the term "antibody."

25 *Monitoring expression of viral genomes*

The invention provides methods for determining the relative fitness of at least two viruses, comprising detecting the level of expression of a first and at least a second reporter sequence, wherein the expression of a reporter sequence is dependent on the fitness of the recombinant virus; and, comparing the level of expression of the first and at least second
 30 reporter sequences, whereby a relatively higher expression of one reporter sequence over the other report sequence indicates greater fitness of that virus having the relatively higher expressed reporter sequence. In one aspect, the method provides for specific detection of the at least two viruses within any mixture, e.g., *in vitro* or *in vivo* system, such as a cell or cell lysate

system. This increased or decreased expression of reporter sequence can be traced by determining the amount of polypeptide present (or nucleic acid present) or by activity assays, depending on the nature of the “detectable moiety” used as a reporter sequence. Polypeptides, peptides and amino acids can be the “detectable moiety”, and can be detected and quantified by any method known in the art, including, e.g., nuclear magnetic resonance (NMR), spectrophotometry, radiography (protein radiolabeling), electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, various immunological methods, e.g. immunoprecipitation, immunodiffusion, immuno-electrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immuno-fluorescent assays, gel electrophoresis (e.g., SDS-PAGE), staining with antibodies, fluorescent activated cell sorter (FACS), pyrolysis mass spectrometry, Fourier-Transform Infrared Spectrometry, Raman spectrometry, GC-MS, and LC-Electrospray and cap-LC-tandem-electrospray mass spectrometries, and the like. Polypeptides (or expressed nucleic acids) also can be detected and/or measured using a protein or nucleic acid array.

Kits

The invention provides kits comprising the compositions, e.g., chimeric nucleic acids, expression cassettes, vectors, cells and/or polypeptides of the invention. The kits also can contain instructional material teaching the methodologies and industrial uses of the invention, as described herein.

The following examples are offered to illustrate but not to limit the invention.

EXAMPLES

Example 1: HIV Reporter Constructs and Viral Fitness Assays

The following example describes exemplary HIV-1 reporter nucleic acid (gene) constructs of the invention and methods of using them, including exemplary HIV viral fitness assays of the invention.

The example describes the exemplary reporter nucleic acid construct based on the small murine heat stable antigen (HSA) protein, which is expressed on the surface of virally infected cells. In this exemplary aspect, an HSA reporter is inserted in the *vpr* reading frame of HIV, leaving *nef* intact. Nine amino acids from the extracellular domain of HSA are replaced with an influenza hemagglutinin (HA) antibody epitope (HSA-HA). Thus, this exemplary

reporter nucleic acid construct is a chimeric nucleic acid construct comprising a nucleotide sequence encoding a heat stable antigen (HSA) reporter sequence comprising a detectable heterologous (non-HSA) marker sequence, where the detectable heterologous marker sequence is disposed within part of a deleted extracellular domain sequence of the HSA reporter sequence.

5 This exemplary reporter nucleic acid construct, being just over 200 nucleotides, is genetically stable in a SCID-Hu mouse model. This exemplary reporter nucleic acid construct has been inserted into the *vpr* reading frame (versus, e.g., in one aspect, *nef*, to allow the study of Nef effects). In this exemplary reporter nucleic acid construct, the HSA reporter protein is conveniently expressed on the surface of infected cells, allowing specific tagging of these cells
10 by immunofluorescent staining for flow cytometry.

This example describes the production of an exemplary reporter gene of the invention using the HSA backbone modified to contain an influenza hemagglutinin (HA) antibody epitope that is expressed on the surface of infected cells, ablating the HSA antibody epitope. This exemplary construct has an advantage of being a small reporter construct - in fact,
15 it (and other constructs of the invention) is a recombinant virus having the same "viral fitness" as comparable wild type virus. This exemplary construct also is clearly distinguishable from an "unmodified" HSA reporter (it can have one or more distinguishable reporter sequences, thus allowing unique identification of each different virus inserted into a growth fitness competition assay), and thus is more versatile than an "unmodified" HSA reporter. Use of this exemplary
20 reporter construct in conjunction with a "parental" unmodified HSA reporter, or another reporter construct of the invention, offers at least a pair of closely matched, yet distinguishable, reporter viruses suitable for a variety of applications, including virus competition assays.

HIV-1 molecular clones and virus stocks. The HIV-1 proviral genome was manipulated in p83-2.1 and p83-10 half-genome plasmids containing the NL4-3 molecular
25 clone, as previously described in, e.g., Ali (2003) J. Virol. Methods 110:137-142; Yang (2003) J. Exp. Med. 197:1365-1375. A plasmid containing murine CD24 (HSA) inserted in the *vpr* reading frame of HIV-1 NL4-3, NL-r-HSAS (see, e.g., Jamieson (1998) supra), was the gift of Dr. Beth Jamieson. A p83-10 plasmid containing the Nef methionine to alanine mutation at position 20 (M20A) was produced by standard PCR mutagenesis (through an AT to GC
30 mutation at NL4-3 nucleotides 8844-8845). Virus stocks were produced by co-electroporation of half-genome plasmids after linearization by Eco RI, and titered by limiting dilution analysis on C8166 cells as previously described, e.g., by Johnson (1990), p. 92-94. In A. Aldovini and B. D. Walker (ed.), Techniques in HIV research. Stockton Press, New York.

Production of half genome HIV-1 plasmids containing the HSA reporter gene in the *vpr* and *nef* reading frames. The p83-2.1/HSA plasmid (containing HSA inserted in the *vpr* reading frame of p83-2.1) was constructed by swapping a PflMI-EcoRI fragment (nucleotides 5297–5743) from NL-r-HSAS into the p83-2.1 half genome plasmid. The p83-10/HSA plasmid (containing HSA inserted in the *nef* reading frame of p83-10) was constructed by first creating an Xba I site in the p83-10 plasmid (corresponding to positions 8781-8785 of NL4-3) by changing nucleotides TATAAG (SEQ ID NO:2) to TCTAGA (SEQ ID NO:3). The HSA gene in NL-r-HSAS was PCR-amplified with an upstream primer containing an Xba I site and a downstream primer containing a Kpn I site, followed by swapping of this product into p83-10 after Xba I and Kpn I digestion. The final constructs are illustrated in Figure 1, showing VPR (SEQ ID NO:4), VPR-HSA (SEQ ID NO:5) and VPR-HSA-HA (SEQ ID NO:1) reporter constructs in the *vpr* locus of HIV-1. Nucleotide sequences for the parental p83-2.1 plasmid (containing the upstream half genome of NL4-3) and the two reporter constructs are shown, starting at 35 nucleotides upstream of the *vpr* start codon. The sequences end with the EcoRI site marking the end of HIV-1 sequences in the plasmid (within the reading frame of *vpr*). “*” indicates no nucleotide. “-” indicates same nucleotide relative to p83-2.1 parental plasmid.

Figure 1 also depicts the HSA reporter gene developed by Drs. Zack and Jamieson (VPR-HSA, SEQ ID NO:5, middle sequence) (Jamieson (1998) *supra*) and an exemplary reporter of the invention, SEQ ID NO:1, VPR-HSA-HA, bottom sequence, as well as locations of PCR primers and probes that can be used to detect each reporter gene specifically (exclusive of the other reporter in a mixture).

Mutagenesis of HSA to create an influenza hemagglutinin antibody epitope (HA).

A nine amino acid antibody epitope (nucleotides TAC CCG TAT GAT GTA CCG GAT TAT GCT (SEQ ID NO:6), amino acids YPYDVPDYA (SEQ ID NO:7) of influenza HA (see, e.g., Wilson (1984) Cell 37:767-778) was inserted in place of nucleotides GCA CCG TTT CCC GGT AAC CAG AAT ATT (SEQ ID NO:8) (amino acids APFPGNQNI) (SEQ ID NO:9) in HSA by site directed mutagenesis using the GENE EDITOR™ Site-Directed Mutagenesis System (Promega, Madison, WI), as per the manufacturer’s protocol. Briefly, the HSA gene was cloned in the pGEM11Zf(+) plasmid for mutagenesis. Following alkaline denaturing, this plasmid was re-annealed to a 5’ phosphorylated oligonucleotide containing desired mutations, followed by synthesis of the mutant strand with DNA polymerase/ligase and transformation into competent *E. coli*. Multiple rounds of mutagenesis, each changing 2 to 6 nucleotides, were

performed to obtain the final product, which was then transferred to the p83-2.1 and p83-10 plasmids following XbaI/KpnI digestion.

Flow cytometric detection of HIV-1-infected cells. T1 cells were infected with reporter viruses at a multiplicity of approximately one (1) tissue culture 50% infectious dose per cell (MOI 1), as previously described Yang (1996) J. Virol. 70:5799-5806; Yang (1997) J. Virol. 71:3120-3128. After infection, equal numbers of infected cells were mixed together and co-cultured for four days. Surface expression of HA and HSA was tested by staining with monoclonal antibodies against HA (Roche, Indianapolis, IN) and/or HSA (BD Pharmingen, San Diego, CA), with or without co-staining of intracellular HIV-1 Gag with the monoclonal antibody KC57 (Coulter, Miami, FL) after permeabilization with CYTOFIX™/CYTOPERM™ (as per manufacturer's protocol, BD Biosciences, San Diego, CA). Flow cytometric analysis was performed on a FACSCAN™ using CELLQUEST™ software running on a G4 POWER™ Macintosh (Becton Dickinson, San Diego, CA).

Real-time PCR quantitation of mixed HIV-1 infected primary PBMC. PBMC were PHA-stimulated (PHA 5µg/ml, Gibco, with IL-2 50U/ml, NIH AIDS Research and Reference Reagent Repository) and separately infected with the HSA and HSA-HA reporter viruses at MOI of 0.005. Two million cells infected with each virus were then co-cultured. At the indicated days after infection, genomic DNA was isolated (DNEASY™, Qiagen, Valencia, CA) for analysis by real time PCR (REAL MASTER MIX™, Eppendorf, Westbury, NY) in a 25µl reaction volume, followed by analysis using an ABI Prism 7700™ (Applied Biosystems, Foster City, CA). HSA and HSA-HA were detected separately using the primers shown in Table 1, below, each multiplexed for β-actin detection, using fluorescence-tagged probes (Integrated DNA technologies, Coralville, IA). Plasmid standards were used to generate curves of C_t versus copy number, after analysis using SDS 1.9.1™ software (Applied Biosystems, Foster City, CA).

Table 1 summarizes primers and probes utilized for real time PCR detection of reporter HIV-1 constructs:

Table 1

Target Gene	Forward Primer	Reverse Primer	Probe
HSA	CGTTTCCCGGTAACCAGA (HSA-specific) (SEQ ID NO:10)	AGAGAGAGAGAGAGCC AGGAGA (HSA-specific) (SEQ ID NO:11)	HEX- TCCCTGCAGTCCACAGCTGG- TAMRA (HSA-specific) (SEQ ID NO:12)
HSA-HA	CCCGTATGATGTACCGGATT (HA-specific) (SEQ ID NO:13)	AGAGAGAGAGAGAGCC AGGAGA (HSA-specific) (SEQ ID NO:14)	HEX- TCCCTGCAGTCCACAGCTGG- TAMRA (HSA-specific) (SEQ ID NO:15)
β -Actin	TCACCCACACTGTGCCCATC TACGA (SEQ ID NO:16)	CAGCGGAACCGCTCATT GCCAATGG (SEQ ID NO:17)	FAM- ATGCCCTCCCCCATGCCATCCTG CGT-TAMRA (SEQ ID NO:18)

Real-time PCR quantitation of mixed HIV-1 infected T1 cells with and without CTL. The T1 cell line (17) was co-infected with NL4-3.1 containing wild type Nef and tagged with HSA-HA in the *vpr* locus, or containing the M20A Nef mutation and tagged with HSA in the *vpr* locus, each at MOI of 0.005. These cells were then cultured with or without the CTL clone 3.23T (7), recognizing A2-restricted epitope SLYNTVATL in Gag (amino acids 77-85) contained in NL4-3.1 (24), at a concentration of 1 CTL per 4 T1 cells. Real time PCR was then performed on days 1 and 4 after infection, as described above.

10 RESULTS

Modification of the murine heat stable antigen (HSA) reporter gene to replace an antibody epitope with the influenza hemagglutinin (HA) epitope: The murine heat stable antigen (HSA) protein has a relatively small extracellular domain (see, e.g., Kay (1991) J. Immunol. 147:1412-1416) containing an epitope recognized by commercially available antibody suitable for immunofluorescent cell surface staining (see, e.g., Jamieson (1998) supra). To produce a matched reporter gene with a distinct antibody epitope, a 27 nucleotide stretch of the extracellular domain of HSA was replaced with 27 nucleotides corresponding to a known epitope of HA (see, e.g., Wilson (1984) supra), see Figure 1, discussed above. Although the exact epitope sequence recognized by the HSA-specific antibody was not known, the intent was to disrupt this epitope while introducing the HA epitope, to allow specific detection of each reporter within a mixture of both.

Specific flow cytometric detection of HIV-1 infected cells with the HSA or HSA-HA reporter. To test whether this exemplary reporter construct of the invention achieved the goal of tagging infected cells and being distinguishable from the parental HSA when stained with cell surface antibodies, whole HIV-1 stocks containing each reporter gene were produced.

Cells were acutely infected with each virus. Co-staining of these infected cells for cell surface HSA or HA and intracellular Gag demonstrated that these reporters were specific for HIV-1-infected cells; reporter-expressing cells also generally stained for intracellular p24, as illustrated in Figure 2.

Figure 2 illustrates data showing detection of HIV-1-infected cells by fluorescent antibody surface staining for the reporter proteins or intracellular Gag. As discussed above, T1 cells were separately infected with HIV-1 containing either an HSA reporter construct or the exemplary HSA-HA reporter construct of the invention (HA in the *vpr* locus). Four days after infection, the cells were surface stained for either reporter in combination with permeabilization and intracellular staining for Gag. Dot plots (gated on live cells by forward and side scatter criteria) are shown. The percentages of cells expressing the four possible combinations of cell surface reporter and intracellular Gag are indicated in the quadrants.

The specificity of staining was further confirmed by mixing cells infected with each reporter virus and co-staining for each reporter protein; each antibody detected a distinct population of cells, as illustrated in Figure 3. The data summarized in illustration of Figure 3 shows the specific detection of cells infected by an HIV-1-HSA reporter construct or the exemplary HIV-1-HSA-HA construct of the invention by fluorescent antibody surface staining. T1 cells were infected as described for Figure 2, above, followed by mixing of infected cells. Four days after infection, the cells were dually-stained for cell surface expression of each reporter; dot plots are shown. The percentages of cells expressing the four possible combinations of the two surface reporters are indicated in the quadrants.

These data indicated that immunofluorescent antibodies against HSA and HA specifically detected cells infected with each virus.

Real-time PCR quantitation of HIV-1 infected cells with the HSA or HSA-HA reporter. To evaluate the utility of these reporter viruses for molecular detection methods, a real-time DNA PCR strategy was developed to measure the viruses and their growth over time. The PCR primer sets included forward primers within the region of the replaced antibody epitope, specific for the replaced "parental" HSA sequence or the inserted HA sequence (i.e., found in the HIV-1-HSA reporter construct or the exemplary HIV-1-HSA-HA construct of the invention, respectively), and a shared reverse primer in HSA, as summarized in Table 1, above, and Figure 4.

Figure 4 illustrates an exemplary real time PCR strategy for specific detection of HIV-1 tagged with the HIV-1-HSA reporter construct or the exemplary HIV-1-HSA-HA

construct of the invention. A schematic representation of each reporter construct within the HIV-1 genome is shown. The approximate locations of primers used for specific amplification of an HSA reporter construct or the exemplary HSA-HA reporter construct of the invention are indicated. Gray shading indicates HSA sequences; black shading indicates the sequences of the inserted HA epitope.

For quantitation, a probe in HSA (capable of amplifying an HSA-encoding sequence) was used to detect either PCR product. This allowed specific quantitation of the “parental” HSA gene or the (modified) exemplary HSA-HA construct of the invention in separate PCR reactions. Through multiplex real-time PCR, detection of either reporter was combined with detection of β -actin (see Table 1, above, for primer sequence) as a common standard. In cultures of primary PBMC infected with two viruses containing these reporters, the growth curve of each virus (expressed as HIV-1 DNA genomes per copies of β -actin DNA over time) could be traced by specific detection of each virus over time, as illustrated in Figure 5.

Figure 5 is a graph illustrating data showing the growth of HIV-1 with the HIV-1-HSA reporter construct or the exemplary HIV-1-HSA-HA construct of the invention in PBMC. PHA-stimulated PBMC were co-infected with the HSA-HA construct or the exemplary HSA-HA reporter virus, and real time DNA PCR quantitation of each reporter in combination with β -actin was performed on days one and four after infection. Infected cell concentration at each time point was calculated as copies of HIV-1 DNA per million copies of β -actin DNA. The $\log_{10}$ units of infected cell concentrations are plotted for each virus at each time point.

NL4-3.1 virus tagged with HSA in the *vpr* locus grew similarly to that tagged with HSA-HA, increasing by about 0.8 to 0.9 $\log_{10}$ units per day. These data indicated that HIV-1 tagged with the HSA and HSA-HA reporter constructs have similar growth characteristics, and are suitably matched for competition assays to determine the relative growth of viruses.

Measurement of relative fitness of HIV-1 strains under a selective constraint. To evaluate whether this real time PCR strategy could detect a difference in the growth of two HIV-1 strains under different selective pressure, we compared the growth of NL4-3.1 containing wild type Nef (WT) versus that containing a methionine to alanine point mutation at position 20 of Nef (M20A) in the presence of HIV-1-specific cytotoxic T lymphocytes (CTL). This mutation had been shown previously to render HIV-1-infected cells more susceptible to CTL by selectively ablating the ability of Nef to downregulate the major histocompatibility class I complex on the surface of infected cells (see, e.g., Tomiyama (2002) J. Virol. 76:7535-7543).

Lymphocytes were infected with WT and M20A viruses tagged with the exemplary HSA-HA recombinant virus and a HSA reporter construct, respectively, and cultured with or without Gag-specific CTL. Real time PCR quantitation of virus growth in these mixed cultures, as illustrated in Figure 6, revealed similar growth of both viruses in the absence of CTL (0.82 log₁₀ units/day and 0.93 log₁₀ units/day, with a ratio of 0.89 for M20A to WT), but different growth rates in the presence of CTL (0.17 log₁₀ units/day and 0.70 log₁₀ units/day, with a ratio of 0.24 for M20A to WT).

Figure 6 graphically illustrates data showing the relative growth of HIV-1 containing wild type or mutated Nef in the absence or presence of HIV-1-specific CTL. HSA-HA-tagged HIV-1 NL4-3.1 containing wild type Nef (WT) and HSA-tagged virus with mutated Nef (M20A, known to render infected cells more sensitive to CTL) were used to co-infect T1 cells. These cells were then cultured in the absence or presence of CTL at a low concentration (1 CTL per 4 T1 cells). Real time PCR quantitation of each virus was then performed as described in Figure 5. Similar results were obtained in a repeat experiment, and the relative impairment of M20A versus WT growth correlated to the concentration of CTL.

These data indicated that while the wild type (WT) and M20A viruses had similar replicative capacity, M20A was disproportionately suppressed by the CTL in comparison to WT, consistent with the known effects of this mutation on inhibition of HIV-1 by CTL (see, e.g., Tomiyama (2002) supra). Overall, our findings demonstrated that the use of this exemplary method of the invention, using paired reporter constructs (including a construct of the invention), allows discrimination of tagged viruses for analysis of relative growth capacity in competition assays.

Measuring relative growth of viruses in competition assays. The relative growth of an immune-resistant versus immune-sensitive strain of HIV was measured in an exemplary competition assay of the invention to determine the impact of the immune response on viral growth. The Nef protein of HIV makes the virus resistant to the cellular immune response, allowing the virus to evade it. However, a simple mutation in Nef (M20A) destroys this function, making it more sensitive to the cellular immune response. The strategy of this study was to compare the fitness of a resistant index virus (WT) against a sensitive test virus (M20A). These viruses grow the same in the absence of any immune response, but M20A grows less if there is an immune response, because it is more susceptible than WT.

Figure 7 shows the result of an experiment where different amounts of immune cells (CTL) were added to some infected cells, to simulate different amounts of immune activity.

The plot shows the fitness ratio (determined as in Figure 6) plotted on the Y-axis, for different amounts of immune cells on the X-axis. The plot demonstrates that when there are very few CTL (left on the X-axis) the ratio is near 1, but when there are more CTL (further right on the X-axis) the ratio decreases. In other words, the ratio of M20A to WT growth reflects how much immune pressure is applied (similar growth if no immune pressure, decreasing less than 1 for more immune pressure). This is an artificial experiment where the immune pressure was artificially manipulated by spiking the infected cells with immune cells (CTL). In one aspect, the assay also is applied to blood cells from patients, where the amount of immune pressure is unknown; the growth ratio would indicate the amount of immune pressure.

Figure 7 shows the growth of the two viruses in mixed cultures over time, as quantitated by real time PCR for each virus (using the primers and probes show in Figure 1 and Table 1). The number of each virus DNA copies is normalized against DNA copies of the cellular gene actin, to reflect the concentration of each virus in the culture. Blood lymphocytes were infected with two identical HIV strains differing only in their reporter (HSA or HSA-HA) and the amount of virus was detected on days 1 and 4. The slopes of the plots are similar (about 0.8 and 0.9 log units per day), meaning the viruses grew at the same rate (ratio of about 1).

Figure 8 shows screening of 11 different clones of HIV for the fitness impact of point mutations in the Gag protein. This was done the same method as Figure 7. Each bar represents the ratio of the growth slopes of a mutant virus (containing a Gag mutation) to the growth slope of a competing index virus (containing a fixed Gag sequence). The first 2 bars of each set show measurements in two independent experiments; the third bar shows the mean and standard deviation of those two experiments. Note that a ratio of 1 means equivalent growth, less than 1 means the mutant grew less well than the index virus, more than 1 means the mutant grew better. It is clear from this assay that mutant 7 reproducibly grew poorly compared to the index virus; most of the others had similar fitness (ratio near 1).

Discussion: Individual tagging of different viruses, e.g., different mutations, substrains, clades or species of HIV-1, as done by exemplary recombinant viruses of the invention, for specific detection is a growth competition assay (e.g., a viral fitness assay of the invention) is a powerful tool for studies of viral replication. In this described aspect, the invention comprises use of a small reporter gene, murine CD24, or heat stable antigen (HSA), inserted into (in place of) a deleted HIV gene sequence whose absence does not affect viral fitness - the accessory gene *vpr* reading frame (and preserving the native reading frame of *nef*). This exemplary construct is relatively stable within the viral genome. We describe the design

and construction of an exemplary reporter gene expressing an influenza hemagglutinin (HA) antibody epitope. This novel exemplary chimeric reporter protein is distinct from the “parental” HSA in that it lacks some native sequence, which is replaced therefor by sequence heterologous to the virus that can be used as a unique, distinguishable marker sequence for that virus. This
5 exemplary chimeric reporter protein is matched in size with the “wild type” sequence to help avoid generating artifactual results in a viral fitness (e.g., growth competition) assay.

Use of a closely matched set of reporters in this exemplary viral fitness (e.g., growth competition) assay of the invention offers the opportunity for controlled comparisons of viral, e.g., HIV-1, strains, mutants, clades and the like. An alternative exemplary application of
10 the methods of the invention comprises measurement of viral replicative fitness. Replicative fitness has been shown to be an important clinical parameter that influences the course of disease, and viral fitness assays are currently being developed as tools for monitoring antiviral drug effects as a result of escape mutation in patients infected with HIV-1 (see, e.g., Bates (2003) Curr. Opin. Infect. Dis. 16:11-18; Nijhuis (2001) Curr. Opin. Infect. Dis. 14:23-28).

The constructs and methods of the invention allow for analysis of the entire viral replicative cycle. Thus, constructs and methods of the invention include consideration of the contribution of all viral genes, e.g., in the example of HIV - reverse transcriptase and protease, to viral replicative capacity. As new drugs are developed to target various genes in the viral life cycle, the constructs and methods of the invention can be used to effectively measure the fitness
15 effects of mutations, deletions or modifications in any gene or nucleic acid segment of a virus.

Thus, the invention provides methods for determining the relative fitness of at least two viruses under a selective constraint or a particular growth condition. Thus, the invention also provides methods for determining the net fitness of at least two different viruses (e.g., two different HIV-1 viruses) under a selective pressure, such as immune cells (e.g., T
25 cells), anti-retroviral drugs, different environmental and growth conditions, and the like. Finally, the small size of “matched” reporters used in the methods of the invention also offer the opportunity to perform viral comparisons within *in vivo* model systems (in addition to *in vitro* systems), such as SCID-Hu mice and/or non-human primates, e.g., chimpanzees. As noted above, using recombinant chimeric viruses of the invention in the methods of the invention
30 improve genetic stability of the reporter construct and allow preservation of all genes important in a virus’s fitness, e.g., allow preservation of an intact *nef* reading frame.

However, a chimeric nucleic acid construct of the invention, e.g., a nucleotide sequence encoding a heat stable antigen (HSA) reporter sequence comprising a detectable

For example, in one aspect, a chimeric nucleic acid construct of the invention inserted into a *nef* gene. We have tested several (HIV) Gag point mutants using reporters in Nef, in fitness competition assays (of the invention, as described herein) against wild type Gag. These assays yielded reproducible measurements of Gag viral mutant fitness as compared to wild type.

(1) NEF

(2) NEF-HSA

37

GGATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTTCATCACGTG
GCCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCTGA (SEQ ID NO:20)

(3) NEF-HSA-HA

5 TATAGAAGTATTACAAGCAGCTTATAGAGCTATTCGCCACATACCTAGAAGAATAAGACAGGGC
TTGGAAAGGATTTTGCTCTAGAAATG*****

*****GGCAGAGCGATGGTGG
10 CCAGGCTAGGGCTGGGGTTGCTGCTTCTGGCACTGCTCCTACCCACGCAGATTTACTGCAACCA
AACATCTGTTTACCCGCTATGATGTACCGGATTATGCTTCTGCTTCCCCAAATCCAAGTAACGCT
ACCACCAGAGGGGGTGGCAGCTCCCTGCAGTCCACAGCTGGTCTCCTGGCTCTCTCTCTCTC
TTCTACATCTCTACTGTTAGGGTACCTTTAAGACCAATGACTTACAAGGCAGCTGTAGATCTTA
GCCACTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAA'TTCACTCCCAAAGAAGACAAGATAT
15 CCTTGATCTGTGGATCTACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGG
CCAGGGGTCAGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATA
AGGTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAAT
GGATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTTCATCACGTG
GCCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCTGA (SEQ ID NO:21)

20

WHAT IS CLAIMED IS:

1. A chimeric nucleic acid construct comprising:
a nucleotide sequence encoding a heat stable antigen (HSA) reporter sequence
comprising a detectable heterologous (non-HSA) marker sequence, wherein the detectable
5 heterologous marker sequence is disposed within or in place of sequence encoding all or part of
the extracellular domain of the heat stable antigen (HSA) reporter sequence.
2. The chimeric nucleic acid construct of claim 1, wherein the reporter
sequence encodes a human or a murine heat stable antigen (HSA) surface protein.
- 10 3. The chimeric nucleic acid construct of claim 1, wherein the detectable
heterologous sequence encodes an epitope recognized by an antibody.
4. The chimeric nucleic acid construct of claim 3, wherein the epitope is
15 derived from a viral polypeptide or peptide,
and optionally the viral polypeptide comprises an influenza hemagglutinin (HA)
polypeptide, and optionally the epitope replaces an antibody epitope in HSA, allowing separate
antibody-based detection of both HSA and HA.
- 20 5. The chimeric nucleic acid construct of claim 1, wherein the reporter
sequence is the same size or substantially the same size as a viral reading frame, wherein
optionally the viral reading frame comprises an HIV-1 *nef* or a HIV-1 *vpr* reading frame.
6. A chimeric nucleic acid comprising a sequence as set forth in SEQ ID
25 NO:1 (VPR-HSA-HA) or SEQ ID NO:21 (Nef-HSA-HA).
7. A chimeric polypeptide encoded by the chimeric nucleic acid construct of
claim 1 or the chimeric nucleic acid of claim 6.
- 30 8. A recombinant virus comprising a chimeric nucleic acid construct as set
forth in claim 1, wherein optionally a sequence of the virus is deleted and the chimeric nucleic
acid construct is inserted into the deleted region of the virus.

9. The recombinant virus of claim 8, wherein
the virus is a lentivirus, and optionally the lentivirus is a human
immunodeficiency virus; or

the virus is from the family *Alphaviridae*, *Flaviviridae*, *Hepadnaviridae*,
5 *Papovaviridae*, *Parvoviridae*, *Herpesviridae*, *Poxviridae*, *Paramyxoviridae*, *Rhabdoviridae* or
Retroviridae.

10. The recombinant virus of claim 8, wherein the human immunodeficiency
virus comprises a human immunodeficiency virus type 1 (HIV-1) or HIV-2.

10

11. The recombinant virus of claim 8, wherein the deleted region of the virus
comprises (i) a reading frame of a non-essential gene or a gene that does not effect the *in vivo*
viability or replication (infectivity) of the virus, or (ii) a section of the viral genome that does not
effect *in vivo* viability or replication (infectivity) of the virus,

15

and the chimeric nucleic acid construct is inserted into the region of the deleted
region and/or in replacement for the deleted region of (i) or (ii).

12. The recombinant virus of claim 11, wherein the entire non-essential gene
region is deleted and the chimeric nucleic acid construct is inserted into and/or in replacement
20 for the deleted region.

13. The recombinant virus of claim 11, wherein the inserted chimeric nucleic
acid construct is substantially or exactly the same size as the deleted region.

25

14. The recombinant virus of claim 11, wherein the deleted region of the virus
comprises a *vpr* reading frame or a *nef* reading frame and the chimeric nucleic acid construct is
inserted into and/or in replacement for the *vpr* reading frame or the *nef* reading frame.

30

15. The recombinant virus of claim 14, wherein all or substantially all of the
vpr region is deleted and the inserted chimeric nucleic acid construct is the same size or
substantially the same size as the deleted sequence.

16. The recombinant virus of claim 8, wherein the heat stable antigen (HSA) reporter sequence encodes a murine heat stable antigen (HSA) lacking all or part of the extracellular domain.

5 17. The recombinant virus of claim 8, wherein the detectable heterologous marker sequence encodes an epitope recognized by an antibody.

18. The recombinant virus of claim 16, wherein the epitope is derived from a viral polypeptide or peptide, and optionally the viral polypeptide comprises an influenza
10 hemagglutinin (HA) polypeptide.

19. A recombinant virus comprising a heterologous sequence comprising a nucleic acid sequence as set forth in SEQ ID NO:1 (Vpr-HSA-HA) or SEQ ID NO:21 (Nef-HSA-HA), wherein the heterologous sequence is inserted into a deleted region of the virus, and
15 the deleted region of the virus comprises (i) a reading frame of a non-essential gene or a gene that does not effect the *in vivo* viability or replication (infectivity) of the virus, or (ii) a section of the viral genome that does not effect *in vivo* viability or replication (infectivity) of the virus.

20. The recombinant virus of claim 19, wherein the virus comprises a human
20 immunodeficiency virus and the deleted region comprises a *vpr* or a *nef* reading frame.

21. A method for determining the relative fitness of at least two viruses, comprising:

(a) introducing a first recombinant virus comprising a first reporter sequence into
25 a host cell, and introducing at least a second recombinant virus comprising at least a second reporter sequence into the host cell, wherein each reporter sequence is distinguishably detectable from the other reporter sequences;

(b) culturing the host cell;

(c) detecting the level of expression of the first and the at least second reporter
30 sequences, wherein the expression of a reporter sequence is dependent on the fitness of the recombinant virus; and,

(d) comparing the level of expression of the first and at least second reporter sequences, whereby a relatively higher expression of one reporter sequence over the other report

sequence indicates greater fitness of that virus having the relatively higher expressed reporter sequence.

22. A method for determining the relative fitness of at least two viruses under a selective constraint or a particular growth condition, comprising:

(a) introducing a first recombinant virus comprising a first reporter sequence into a host cell, and introducing at least a second recombinant virus comprising at least a second reporter sequence into the host cell, wherein each reporter sequence is distinguishably detectable from the other reporter sequences;

(b) culturing the host cell in the presence or absence of a selective constraint, or in at least two different growth conditions;

(c) detecting the level of expression of the first and the at least second reporter sequences, wherein the expression of a reporter sequence is dependent on the fitness of the recombinant virus; and

(d) comparing the expression of the reporter sequences in the presence or absence of the selective constraint or growth condition, whereby the virus with the greatest fitness has an increased expression of its reporter sequence in the presence of the selective constraint or the particular growth condition.

23. The method of claim 22, wherein the selective constraint comprises virus-specific lymphocytes,

and optionally the lymphocytes are specific for a wild type strain of the virus, and optionally the lymphocytes are virus-specific T cells.

24. The method of claim 22, wherein the selective constraint comprises an antiviral agent, wherein optionally the antiviral agent comprises an antiviral drug or antiviral antibody.

25. The method of claim 22, wherein the at least two different growth conditions comprises culturing the host cell in alternative *in vitro* culture environments, *in vivo* environments, temperatures or pHs.

26. A method for determining the effectiveness of a viral vaccine, comprising:

(a) introducing a first recombinant virus comprising a first reporter sequence into a host cell *in vitro* or *in vivo*, wherein the first virus is immune resistant, and introducing at least a second recombinant virus comprising at least a second reporter sequence into the host cell *in vitro* or *in vivo*, wherein the at least a second virus is immune sensitive;

(b) culturing the host cells *in vitro* or *in vivo* in the presence or absence of peripheral blood mononuclear cells (PBMC) isolated from a patient inoculated with a vaccine against the virus;

(c) determining the level of expression of the first and the at least second reporter sequences, wherein the expression of each reporter sequence is dependent on the relative fitness of the recombinant virus; and

(d) comparing the expression of the reporter sequences in the presence or absence of the PBMC, whereby the ratio of recombinant virus growth as indicated by reporter sequence expression reflects the effectiveness of the vaccine at eliciting a biologically relevant anti-viral immune response,

wherein optionally the first and at least second recombinant virus are members of different strains, substrains or clades of the virus,

and optionally the first and at least second recombinant virus are members of the same strain, substrain or clade of the virus, but differ in nucleic acid sequence or viral protein expression.

27. A method for determining the relative effectiveness of a viral vaccine against at least two different viruses, the method comprising the steps of:

(a) introducing a first recombinant virus comprising a first reporter sequence into a host cell *in vitro* or *in vivo*, and introducing at least a second recombinant virus comprising at least a second reporter sequence into the host cell *in vitro* or *in vivo*,

wherein optionally the first and at least second recombinant virus are members of different strains, substrains or clades of the virus,

and optionally the first and at least second recombinant virus are members of the same strain, substrain or clade of the virus, but differ in nucleic acid sequence or viral protein expression;

(b) culturing the host cells *in vitro* or *in vivo* in the presence or absence of peripheral blood mononuclear cells (PBMC) or antibodies isolated from an individual inoculated with a vaccine directed against the virus;

(c) determining the level of expression of the first and the at least second reporter sequences, wherein the expression of each reporter sequence is dependent on the relative fitness of the recombinant virus; and

(d) comparing the expression of the reporter sequences in the presence or absence of the PBMC or antibodies, whereby the ratio of virus growth as indicated by reporter sequence expression reflects the effectiveness of the vaccine at eliciting a biologically relevant anti-viral immune response.

28. A method for determining the relative effectiveness of a test compound or an anti-viral drug against at least two different viruses, the method comprising the steps of:

(a) providing at least one test compound or anti-viral drug;

(b) providing at least two different recombinant viruses each comprising a detectably different reporter sequence, and a host cell compatible with the growth of the at least two different recombinant viruses,

wherein optionally the first and at least second recombinant virus are members of different strains, substrains or clades of the virus,

and optionally the first and at least second recombinant virus are members of the same strain, substrain or clade of the virus, but differ in nucleic acid sequence or viral protein expression;

(c) introducing a first recombinant virus comprising a first reporter sequence into the host cell, and introducing at least a second recombinant virus comprising at least a second reporter sequence into the host cell;

(d) culturing the host cells in the presence or absence of the test compound or anti-viral drug;

(e) determining the level of expression of the first and the at least second reporter sequences, wherein the expression of each reporter sequence is dependent on the relative fitness of the recombinant virus; and

(f) comparing the expression of the reporter sequences in the presence or absence of the test compound or anti-viral drug, whereby the ratio of virus growth as indicated by

reporter sequence expression reflects the effectiveness of the test compound or anti-viral drug to elicit an anti-viral response against the first and at least second virus.

29. The method of any of the claims 21 to 28, wherein the first and or at least
5 second recombinant virus comprises a recombinant virus as set forth in any of claims 8 to 18.

30. The method of claim 29, wherein the first reporter sequence comprises a
chimeric a heat stable antigen (HSA) reporter sequence and the at least second reporter sequence
comprises a heat stable antigen (HSA) reporter sequence; and optionally the first reporter
10 sequence comprises SEQ ID NO:1 (HSA-HA) and the at least second reporter sequence
comprises an HSA.

31. The method of any of the claims 21 to 28 , wherein the first or the at least
second reporter sequence encodes a heat stable antigen comprising a heterologous detectable
15 domain in or in place of all or part of an extracellular domain sequence;

wherein optionally the all or a portion of the extracellular domain is deleted and
the heterologous detectable domain is the same size or substantially the same size as the deleted
sequence, and optionally the heat stable antigen is a murine heat stable antigen or a human heat
stable antigen.

32. The method of any of the claims 21 to 31, wherein level of reporter
sequence expression is assessed or determined by RNA message or protein expression detection
methods, and optionally the reporter sequence is expressed on the extracellular surface of the
host cell.

33. The method of claim 32, wherein the level of reporter sequence
expression is determined by flow cytometric analysis, and optionally the flow cytometric
analysis is by Fluorescence Activated Cell Sorter (FACS).

34. The method of claim 32, wherein the level of reporter sequence
expression is determined by molecular detection methods.

35. The method of claim 34, wherein the level of reporter sequence expression is determined by polymerase chain reaction (PCR), wherein optionally the PCR comprises real-time PCR.

5 36. The method of any of the claims 21 to 31, wherein the virus is a retrovirus, a hepadnavirus, a flavivirus, or a herpesvirus.

37. The method of claim 36, wherein the virus is human immunodeficiency virus (HIV) and optionally the human immunodeficiency virus (HIV) is HIV-1 or HIV-2.

10 38. The method of claim 22, claim 26, claim 27 or claim 28, wherein the recombinant viruses are introduced into the host cells *in vitro*.

15 39. The method of claim 38, wherein the recombinant viruses are introduced into the host cells *in vitro*, and the host cells are subsequently implanted or transferred *in vivo* to a non-human animal, and optionally the non-human animal is a mouse, a rat or a non-human primate.

20 40. The method of claim 22, claim 26, claim 27 or claim 28, wherein the recombinant viruses are introduced into the host cells *in vivo*.

25 41. The method of claim 38, wherein the recombinant viruses are introduced *in vivo* into host cells in a non-human animal, and optionally the non-human animal is a mouse, a rat or a non-human primate.

42. The method of claim 39 or claim 41, wherein the non-human animal is a SCID-Hu mice or a chimpanzee.

30 43. A host cell comprising a recombinant virus as set forth in any of claims 8 to 18, a chimeric nucleic acid construct as set forth in any of claims 1 to 6, or a chimeric polypeptide as set forth in claim 7.

44. A transgenic non-human animal comprising a recombinant virus as set forth in any of claims 8 to 18, a chimeric nucleic acid construct as set forth in any of claims 1 to 6, or a chimeric polypeptide as set forth in claim 7.

5 45. A kit comprising a recombinant virus as set forth in any of claims 8 to 18, a chimeric nucleic acid construct as set forth in any of claims 1 to 6, or a chimeric polypeptide as set forth in claim 7, wherein optionally the kit comprises instructions to practice a method of any of the claims 21 to 28.

10 46. A pair of recombinant viruses, wherein at least one member of the pair comprises a recombinant virus as set forth in any of claims 8 to 18, and the pair are related members of the same genera, family, strain, substrain or clade.

FIGURE 1

					<u>Vpr Start</u>		
VPR	CCTTTGCCTA	GTGTTAGGAA	ACTGACAGAG	GACAGATGGA	ACAAGCCCCA	GAAGACCAAG	
VPR-HSA	-----	-----	-----	-----G-----	-----	-----	
VPR-HSA-HA	-----	-----	-----	-----G-----	-----	-----	

					<u>XbaI</u>		
VPR	GGCCACAGAG	GGAGCCATAC	AATGAATGGA	CACTAGAGCT	TTTAGAGGAA	C'TAAGAGTG	
VPR-HSA	-----	-----	-C-----A--	-----	-C-----	****	*****
VPR-HSA-HA	-----	-----	-C-----A--	-----	-C-----	****	*****

VPR	AAGCTGTTAG	ACATTTCCT	AGGA TATGGC	TCCATAACTT	AGGACAACAT	ATCTATGAAA	
VPR-HSA	*****	*****	*****	*****	*****	*****	
VPR-HSA-HA	*****	*****	*****	*****	*****	*****	

VPR	CTTACGGGGA	TACTTGGGCA	GGAG TGAAG	CCATAATAA*	*****	*****	
VPR-HSA	*****	*****	*****	*****G	AGTCGCGCCG	CGCGCCGACG	
VPR-HSA-HA	*****	*****	*****	*****G	AGTCGCGCCG	CGCGCCGACG	

					<u>Reporter Start</u>		
VPR	*****	*****	*****	*****	*****	*****	
VPR-HSA	GAGCGGACAT	GGCAGAGCG	ATGGTGGCCA	GGCTAGGGCT	GGGGTTGCTG	CTTCTGGCAC	
VPR-HSA-HA	GAGCGGACAT	GGCAGAGCG	ATGGTGGCCA	GGCTAGGGCT	GGGGTTGCTG	CTTCTGGCAC	

					<u>Replaced Epitope</u>		
VPR	*****	*****	*****	*****	*****	*****	
VPR-HSA	TGCTCCTACC	CACGCAGATT	TACTGCAACC	AAACATCTGT	TGCACCGTTT	CCCGGTAACC	
VPR-HSA-HA	TGCTCCTACC	CACGCAGATT	TACTGCAACC	AAACATCTGT	TATACCGTAT	GATGTACCGG	

VPR	*****	*****	*****	*****	*****	*****	
VPR-HSA	AGAATATTTC	TGCTTCCCCA	AATCCAAGTA	ACGC TACCAC	CAGAGGGGGT	GGCAGCTCCC	
VPR-HSA-HA	ATTATGCTTC	TGCTTCCCCA	AATCCAAGTA	ACGC TACCAC	CAGAGGGGGT	GGCAGCTCCC	

	<u>Reporter</u>					<u>Stop</u>
VPR	*****	*****	*****	*****	*****	*****
VPR-HSA	TGCAGTCCAC	AGCTGGTCTC	CTGGCTCTCT	CTCTCTCTCT	TCTACATCTC	TACTGTAGTA
VPR-HSA-HA	TGCAGTCCAC	AGCTGGTCTC	CTGGCTCTCT	CTCTCTCTCT	TCTACATCTC	TACTGTAGTA

	<u>EcoRI</u>
VPR	GAATTC
VPR-HSA	-----
VPR-HSA-HA	-----

FIGURE 2

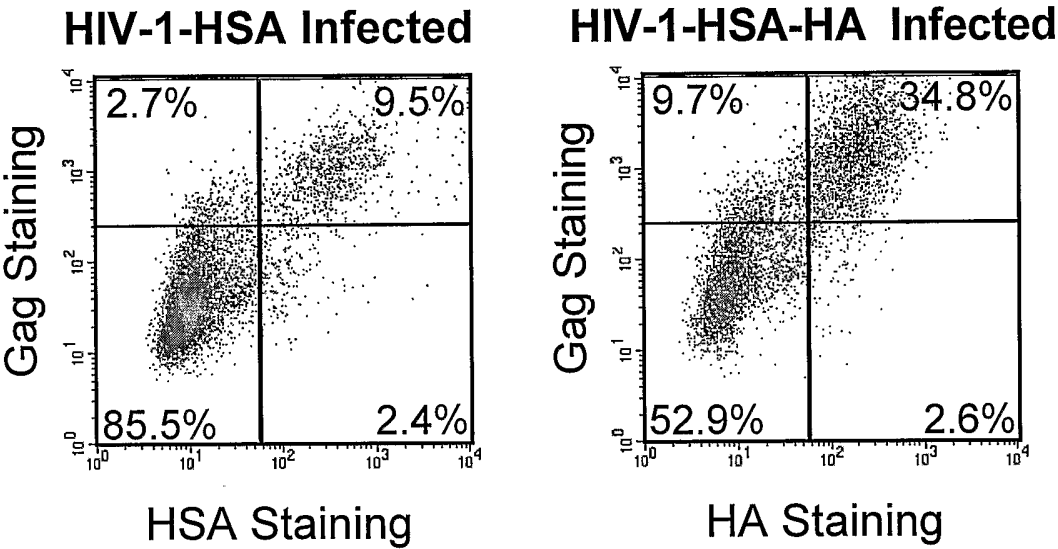


FIGURE 3

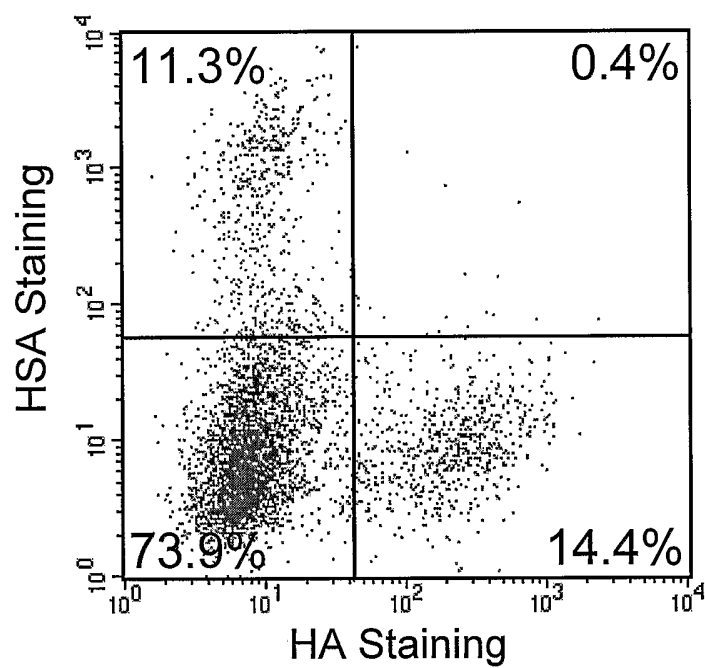


FIGURE 4

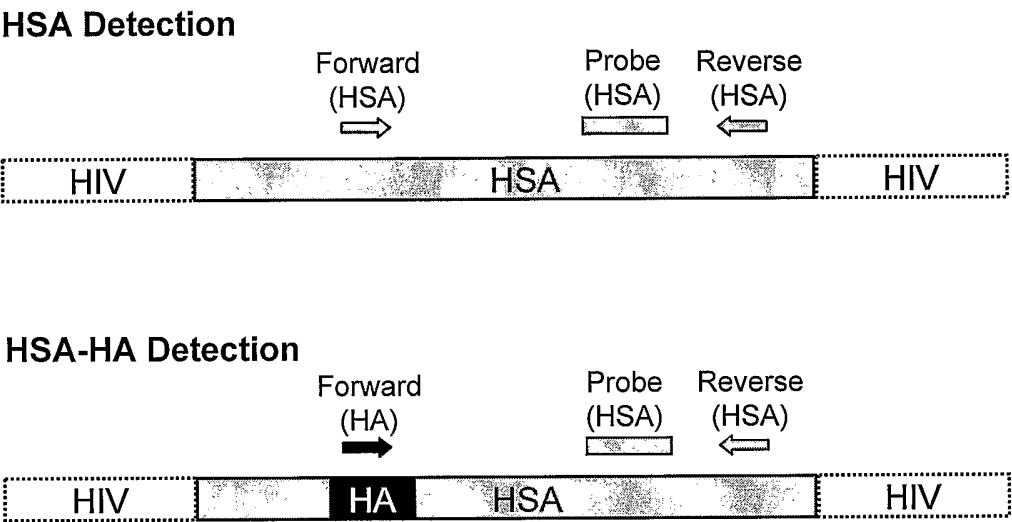


FIGURE 5

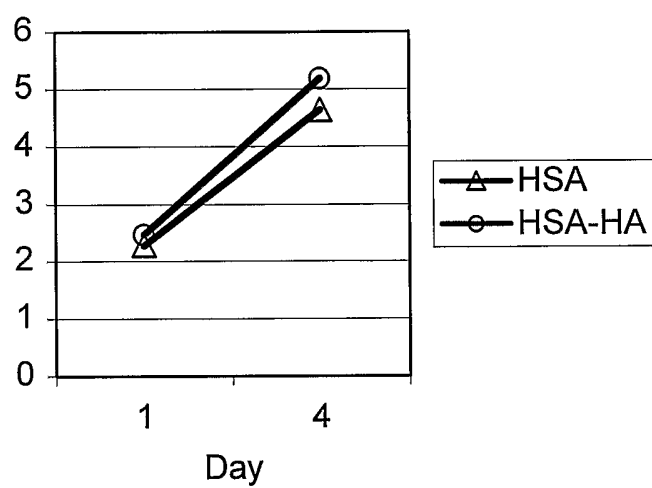


FIGURE 6

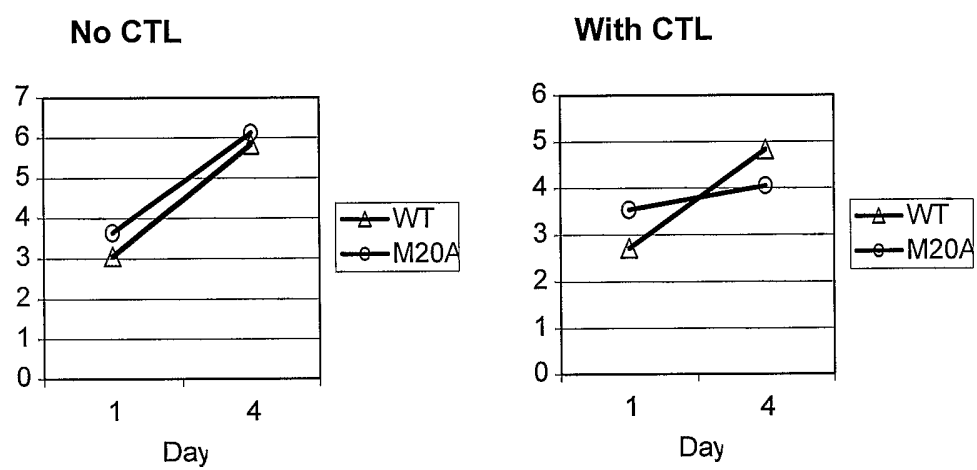


FIGURE 7

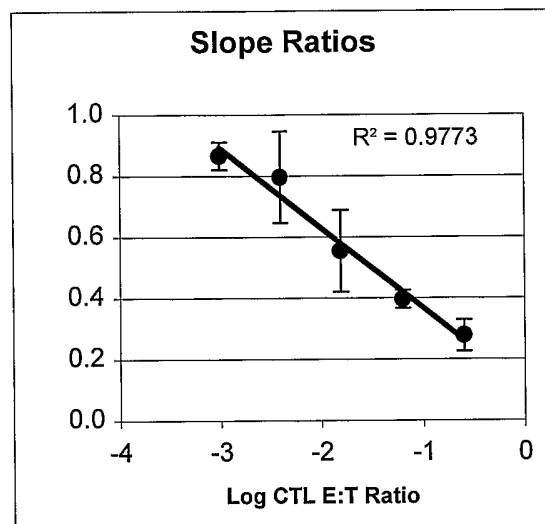


FIGURE 8

