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(54) Title: MEGANUCLEASE VARIANTS CLEAVING THE GENOMIC INSERTION OF A VIRUS AND USES THEREOF

(57) Abstract: Meganuclease variants which cleave the genomic insertion of an integrating Virus genome and in particular to meganuclease variants which cleave the Human Immunodeficiency Virus genome following genomic insertion, known as the HIV provirus. Vector encoding such variants, as well as to a cell or multi-cellular organism modified by such a vector and use of said meganuclease variants and derived products for genome engineering and for in vivo and ex vivo (gene cell therapy) genome therapy.



WO 2010/122367 A2

MEGANUCLEASE VARIANTS CLEAVING THE GENOMIC INSERTION OF A VIRUS AND USES THEREOF

The invention relates to meganuclease variants which cleave the
5 genomic insertion of an integrating Virus genome and in particular to meganuclease
variants which cleave the Human Immunodeficiency Virus genome following
genomic insertion, known as the HIV provirus. The present Invention also relates to a
vector encoding such variants, as well as to a cell or multi-cellular organism modified
by such a vector and to the use of said meganuclease variant and derived products for
10 genome engineering and for *in vivo* and *ex vivo* (gene cell therapy) genome therapy.

Viral infections of various sorts are a serious and continuing health,
agricultural and economic problem worldwide. In particular viruses present specific
treatment and control problems as they always comprise an intracellular stage to their
life cycle, in which the nucleic acid genome of the virus is inserted into a host cell and
15 normally transported to the nucleus. During this stage of the virus life cycle, the virus
genome can enter into a dormant state whilst inside a host cell, in which the
production of new virus particles/proteins/copies of the viral genome ceases. These
characteristics present a significant problem as most medicaments and treatments for
viral infection consist of compounds which affect aspects of virus biology involved in
20 the active stages of the virus life cycle, such as compounds which target a viral
enzyme or structural protein. Therefore whilst in a dormant state the viral genome
resident in the cytoplasm or nucleus of a host cell can not be affected by most
conventional anti-virus medicaments and therefore persists.

One group of viruses presents additional problems as they integrate
25 into the host cell genome. This group, called retroviruses, can be transmitted via the
infection of new host cells by virus particles and can also cause the endemic infection
of the progeny cells of a host cell in which they are genomically integrated. This
second mode of transmission, particularly when the retrovirus genome is dormant can
result in the clonal expansion of the retrovirus containing cells, which in turn can
30 cause significant problems once the retrovirus genomes activate.

The present invention therefore relates to Retroviruses which are
contained with the family Retroviridae which comprises in turn seven genera.

Alpharetrovirus, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, *Lentivirus* and *Spumavirus*. These groups of viruses are responsible for several important diseases such as Human T-lymphotrophic virus (*Gammaretrovirus*), Rous Sarcoma (*Alpharetrovirus*) and Human Immunodeficiency Virus (*Lentivirus*).

5 The Human Immunodeficiency Virus (HIV) (Figure 1) is an example of a Retrovirus which is responsible for a significant and ongoing global medical crisis. HIV viruses persist and continue to replicate for many years in the infected individual before causing overt signs of disease. HIV is the causative agent of the Acquired Immune Deficiency Syndrome (AIDS), which is characterized by a
10 susceptibility to infection with opportunistic pathogens, mainly as a result of a profound decrease in the number of CD4+ T cells. A characteristic feature of the *Retroviridae* family of viruses is that viral particles contain two copies of an RNA genome. After infection, the genomic RNA is reverse transcribed by a viral enzyme into DNA, which is permanently integrated into the host genome.

15 The retroviral genome harbors the sequences coding for the enzymatic, structural, and regulatory proteins. In addition, the genomic RNA molecule contains a series of non-coding sequences that have important functions in different steps of the viral life cycle (Figure 2).

 The “2007 AIDS epidemic update” report, issued by the UNAIDS
20 (Joint United Nations Programme on HIV/AIDS), indicates that 33.2 million [30.6 - 36.1 million] people were estimated to be living with HIV, 2.5 million [1.8 - 4.1 million] people became newly infected and 2.1 million [1.9 - 2.4 million] people died of AIDS in 2007.

 HIV is characterized by a high genetic variability, due to the rapid
25 viral turnover ($10^{10} - 10^{12}$ viral particles produced per day) in an HIV-infected individual, combined with the high mutation rate arising during reverse transcription (10^{-4} per nucleotide). Two types of HIV, HIV-1 and HIV-2, which are closely related to each other, have been identified to date (Sharp et al., Philos Trans R Soc Lond B Biol Sci, 2001, 356, 867-76. Most AIDS worldwide is caused by the more virulent
30 HIV-1, while HIV-2 is endemic in West Africa. Both viruses appear to have spread to humans from other primate species and the best evidence from sequence relationships suggests that HIV-1 has passed to humans on at least three independent occasions

from the chimpanzee, *Pan troglodytes* and HIV-2 from the sooty mangabey, *Cercocebus atys*.

The three zoonotic transmissions that generated the HIV-1 type viruses gave rise to three different viral groups: M, O and N. The M group (for main),
5 represents the substantial majority of worldwide infections. The O (for outlier) and N (for non-M/non-O) groups remains essentially restricted to Central Africa (Sharp et al., Philos Trans R Soc Lond B Biol Sci, 2001, 356, 867-76).

HIV is transmitted by direct sexual contact, by blood or blood products, and from an infected mother to infant, either intrapartum, perinatally, or via
10 breast milk. Infection of humans with HIV-1 causes a dramatic decline in the number of CD4+ T lymphocytes. When the number of CD4+ cells is very reduced, opportunistic infections and neoplasms occur (Simon et al., Lancet, 2006, 368, 489-504).

Antiretroviral treatment for HIV infection consists of drugs which work by slowing down the replication of HIV in the body. Currently, there are around
15 30 antiretroviral drugs approved to treat people infected with HIV in various countries around the world. There are four classes of anti-HIV drugs that attack the virus in a different way. Namely nucleoside or nucleotide reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors and entry inhibitors (Flexner C, Nature Reviews Drug Discovery, 2007, 6, 959-966).

20 People with HIV need to continuously take antiretroviral drugs. Furthermore, for antiretroviral treatment to be effective for a long time, it has been found that more than one antiretroviral drug must be taken at a time. This is known as Combination Therapy. The term Highly Active Antiretroviral Therapy (HAART) is used to describe a combination of three or more anti-HIV drugs. HAART typically
25 combines drugs from at least two different classes of antiretroviral drugs and has been shown to effectively suppress the virus when used properly. Highly active antiretroviral therapy has revolutionized how people infected with HIV are treated, and reduces the rate at which resistance develops.

Normally when anti-HIV treatment is started, the viral load drops to
30 an undetectable level. When drug resistance develops, the amount of HIV in the blood rises and the risk of the person becoming ill increases and this usually means that the

drug regimen needs to be changed (Martinez-Cajas and Wainberg, *Drugs*, 2008, 68 , 43-72).

Currently available HIV treatments have converted HIV infection into a chronic disease, increasing the lifespan of infected individuals. Anti-HIV drugs can reduce the rate of viral replication, retarding therefore the onset of AIDS. Nevertheless, the emergence of strains resistant to these treatments prompt to the development of new therapeutic strategies (Rossi et al., *Nat. Biotechnol.*, 2007, 25, 1444-54). Although there are currently no vaccines to prevent or treat HIV, researchers are developing and testing potential HIV vaccines, either preventive or therapeutic. However, vaccine development encounters the same problem as anti-HIV drugs concerning the rapid viral evolution and the subsequent development of resistance. Even if therapeutic vaccines could be effective, they may be able to keep HIV infection under control. In spite of this, the scientific and medical community agrees that therapeutic HIV vaccines will not be able to completely eliminate HIV infection, because the virus "hides" in certain cells of the body, where it can last silent for decades.

A new field for the treatment of HIV infection is the development of genetic therapies against HIV. Gene therapy could allow preventing progressive HIV infection by persistently blocking viral replication. Gene-targeting strategies are being developed with RNA-based agents (such as ribozymes, aptamers and small interfering RNAs) and protein-based agents. Among the last group, the use of zinc-finger nucleases against the CCR5 receptor, a protein present in the surface of immune cells that is required to mediate viral entry, is on Phase I clinical trial at the moment. In this case, the disruption of the CCR5 receptor from the immune cells by the nucleases is proposed to render the patient's cells permanently resistant to CCR5-specific strains of HIV. This approach is based on the fact that people with natural mutations on this receptor are resistant to HIV infection.

To date however the number of effective anti-HIV/retrovirus therapies is very small, due in part to the limited number of target genes/proteins/pathways present in the relatively simple retrovirus genome/life cycle as well as to the rapid creation of mutants by the retrovirus during replication which allow members of the virus population to evade therapeutic compounds that more

slowly evolving pathogens such as bacteria or protozoa can not develop resistance to quickly enough.

An interesting target that has not already been pursued in the fight against the AIDS pandemic and more generally retroviruses is the genomically integrated provirus, since targeting the proviral DNA could lead to the elimination or inactivation of the structure that allows the virus to multiply and the infection to propagate. One novel way to inactivate the provirus which the inventors have decided to investigate is by the use of nucleases that could cleave the integrated form of the virus and generate mutations and/or deletions after the action of the cellular DNA repair machinery.

An important point to be considered in this kind of approach is the choice of the target sequences that would be cleaved. In a first instance, the target sequences should be located in the coding sequences of essential genes, since the inactivation of an accessory gene would not lead to viral eradication. The viral genome also contains essential regulatory sequences that are located in the long terminal repeats (LTRs) that flank the viral genome in the provirus. Even if mutations in these regions could be less drastic than a mutation in an essential gene, the fact that they are duplicated sequences could be useful in an approach of "virus clipping", meaning the excision of long regions of the proviral DNA by the action of a nuclease cleaving twice in the viral sequence. Another important point that should be considered is the degree of sequence variation that is observed in the target sequences among different circulating viral isolates. Indeed, HIV is characterized by a high degree of sequence variability due to the nature of the viral reverse transcriptase. It is therefore essential to check the sequence conservation of the target among the different isolates that are found nowadays in the pandemic.

The inventors have developed a new molecular medicine approach based on the inactivation of the retrovirus provirus using the HIV-1 provirus in the genome of the infected cell as a model, through the use of tailored meganucleases specifically targeting the proviral DNA. The principle of this new therapeutic strategy is that the tailored meganucleases against targets in the provirus will generate a double strand break (DSB) at their target sequences, chosen to be located in genes/regulatory sequences/structural sequences that are essential for the virus to replicate.

The epidemiology of HIV, particularly in sub Saharan Africa, makes research into the HIV virus a major and extremely active area of research. The manipulation of the HIV provirus is one area of research in which to date reagents have not been readily available as workers have instead concentrated on attempting to
5 manipulate the HIV virion *per se*. Therefore the means to easily engineer the HIV provirus *in situ* in the genome of an infected cell/organism would likely provide valuable insights into this aspect of HIV biology and potentially open new avenues of attack in combating HIV.

Even if the meganuclease targets have been selected following the
10 criteria mentioned above, namely in essential genes and particularly in sequences showing the highest degree of conservation, the capacity of the virus to generate escape mutants under the selective pressure of a drug/therapy must be considered. To minimize the effect of drug resistance(s), "combination" therapy is used. This involves the simultaneous use of drugs which have different modes of action to target
15 the strains present in the patient. As a result, the probability of a virus to simultaneously develop resistance to both drugs, which would need to occur in order for the virus to 'escape' the treatment regime, is very low. In the same way, the possibility of using a combination of meganucleases could help to fight against the potential resistance that could be generated during viral replication.

In vivo meganucleases are essentially represented by homing
20 endonucleases. Homing Endonucleases (HEs) are a widespread family of natural meganucleases including hundreds of proteins families (Chevalier, B.S. and B.L. Stoddard, Nucleic Acids Res., 2001, 29, 3757-3774). These proteins are encoded by mobile genetic elements which propagate by a process called "homing": the
25 endonuclease cleaves a cognate allele from which the mobile element is absent, thereby stimulating a homologous recombination event that duplicates the mobile DNA into the recipient locus. Given their exceptional cleavage properties in terms of efficacy and specificity, they could represent ideal scaffolds to derive novel, highly specific endonucleases.

30 HEs belong to four major families. The LAGLIDADG family, named after a conserved peptide motif involved in the catalytic center, is the most widespread and the best characterized group. Seven structures are now available. Whereas most

proteins from this family are monomeric and display two LAGLIDADG motifs, a few have only one motif, and thus dimerize to cleave palindromic or pseudo-palindromic target sequences.

Although the LAGLIDADG peptide is the only conserved region
5 among members of the family, these proteins share a very similar architecture (Figure 3). The catalytic core is flanked by two DNA-binding domains with a perfect two-fold symmetry for homodimers such as I-*CreI* (Chevalier, *et al.*, Nat. Struct. Biol., 2001, 8, 312-316) , I-*MsoI* (Chevalier *et al.*, J. Mol. Biol., 2003, 329, 253-269) and I-*CeuI* (Spiegel *et al.*, Structure, 2006, 14, 869-880) and with a pseudo symmetry for
10 monomers such as I-*SceI* (Moure *et al.*, J. Mol. Biol., 2003, 334, 685-69, I-*DmoI* (Silva *et al.*, J. Mol. Biol., 1999, 286, 1123-1136) or I-*AniI* (Bolduc *et al.*, Genes Dev., 2003, 17, 2875-2888). Both monomers and both domains (for monomeric proteins) contribute to the catalytic core, organized around divalent cations. Just above the catalytic core, the two LAGLIDADG peptides also play an essential role in the
15 dimerization interface. DNA binding depends on two typical saddle-shaped $\alpha\beta\beta\alpha\beta\beta\alpha$ folds, sitting on the DNA major groove. Other domains can be found, for example in inteins such as PI-*PfuI* (Ichiyanagi *et al.*, J. Mol. Biol., 2000, 300, 889-901) and PI-*SceI* (Moure *et al.*, Nat. Struct. Biol., 2002, 9, 764-770), whose protein splicing domain is also involved in DNA binding.

20 The making of functional chimeric meganucleases, by fusing the N-terminal I-*DmoI* domain with an I-*CreI* monomer (Chevalier *et al.*, Mol. Cell., 2002, 10, 895-905 ; Epinat *et al.*, Nucleic Acids Res, 2003, 31, 2952-62; International PCT Applications WO 03/078619 and WO 2004/031346) have demonstrated the plasticity of LAGLIDADG proteins.

25 Different groups have also used a semi-rational approach to locally alter the specificity of the I-*CreI* (Seligman *et al.*, Genetics, 1997, 147, 1653-1664; Sussman *et al.*, J. Mol. Biol., 2004, 342, 31-41; International PCT Applications WO 2006/097784, WO 2006/097853, WO 2007/060495 and WO 2007/049156; Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; Rosen *et al.*, Nucleic Acids Res., 2006, 34, 4791-4800 ; Smith *et al.*, Nucleic Acids Res., 2006, 34, e149), I-*SceI* (Doyon *et al.*, J. Am. Chem. Soc., 2006, 128, 2477-2484), PI-*SceI* (Gimble *et al.*, J. Mol. Biol., 2003, 334, 993-1008) and I-*MsoI* (Ashworth *et al.*, Nature, 2006, 441, 656-659).

30

In addition, hundreds of I-CreI derivatives with locally altered specificity were engineered by combining the semi-rational approach and High Throughput Screening:

- Residues Q44, R68 and R70 or Q44, R68, D75 and I77 of I-CreI were mutagenized and a collection of variants with altered specificity at positions ± 3 to 5 of the DNA target (5NNN DNA target) were identified by screening (International PCT Applications WO 2006/097784 and WO 2006/097853; Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; Smith *et al.*, Nucleic Acids Res., 2006, 34, e149).

- Residues K28, N30 and Q38 or N30, Y33 and Q38 or K28, Y33, Q38 and S40 of I-CreI were mutagenized and a collection of variants with altered specificity at positions ± 8 to 10 of the DNA target (10NNN DNA target) were identified by screening (Smith *et al.*, Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156).

Two different variants were combined and assembled in a functional heterodimeric endonuclease able to cleave a chimeric target resulting from the fusion of two different halves of each variant DNA target sequence (Arnould *et al.*, precited; International PCT Applications WO 2006/097854 and WO 2007/034262), as illustrates in Figure 4.

Furthermore, residues 28 to 40 and 44 to 77 of I-CreI were shown to form two separable functional subdomains, able to bind distinct parts of a homing endonuclease half-site target sequence (Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/049095 and WO 2007/057781).

The combination of mutations from the two subdomains of I-CreI within the same monomer allowed the design of novel chimeric molecules (homodimers) able to cleave a palindromic combined DNA target sequence comprising the nucleotides at positions ± 3 to 5 and ± 8 to 10 which are bound by each subdomain (Smith *et al.*, Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/049095 and WO 2007/057781).

The combination of the two former steps allows a larger combinatorial approach, involving four different subdomains. The different subdomains can be modified separately and combined to obtain an entirely redesigned meganuclease variant (heterodimer or single-chain molecule) with chosen specificity, as illustrated

in Figure 5. In a first step, couples of novel meganucleases are combined in new molecules (“half-meganucleases”) cleaving palindromic targets derived from the target one wants to cleave. Then, the combination of such “half-meganucleases” can result in a heterodimeric species cleaving the target of interest. The assembly of four
5 sets of mutations into heterodimeric endonucleases cleaving a model target sequence or a sequence from different genes has been described in the following patent applications: XPC gene (WO2007093918), RAG gene (WO2008010093), HPRT gene (WO2008059382), beta-2 microglobulin gene (WO2008102274), Rosa26 gene (WO2008152523) and Human hemoglobin beta gene (WO200913622).

10 The method for producing meganuclease variants and the assays based on cleavage-induced recombination in mammal or yeast cells, which are used for screening variants with altered specificity are described in the International PCT Application WO 2004/067736; Epinat *et al.*, Nucleic Acids Res., 2003, 31, 2952-2962; Chames *et al.*, Nucleic Acids Res., 2005, 33, e178, and Arnould *et al.*, J. Mol.
15 Biol., 2006, 355, 443-458. These assays result in a functional LacZ reporter gene which can be monitored by standard methods.

These variants can be used to cleave genuine chromosomal sequences and have paved the way for novel perspectives in several fields, including gene therapy.

20 Even though the base-pairs ± 1 and ± 2 do not display any contact with the protein, it has been shown that these positions are not devoid of content information (Chevalier *et al.*, J. Mol. Biol., 2003, 329, 253-269), especially for the base-pair ± 1 and could be a source of additional substrate specificity (Argast *et al.*, J. Mol. Biol., 1998, 280, 345-353; Jurica *et al.*, Mol. Cell., 1998, 2, 469-476; Chevalier,
25 B.S. and B.L. Stoddard, Nucleic Acids Res., 2001, 29, 3757-3774). *In vitro* selection of cleavable I-CreI targets (Argast *et al.*, precited) randomly mutagenized, revealed the importance of these four base-pairs on protein binding and cleavage activity. It has been suggested that the network of ordered water molecules found in the active site was important for positioning the DNA target (Chevalier *et al.*, Biochemistry, 2004,
30 43, 14015-14026). In addition, the extensive conformational changes that appear in this region upon I-CreI binding suggest that the four central nucleotides could

contribute to the substrate specificity, possibly by sequence dependent conformational preferences (Chevalier *et al.*, 2003, precited).

Therefore the inventors seeing the problems associated with retro-viruses and in particular HIV, have generated a new class of reagents which can be
5 used to specifically target and manipulate the retroviral provirus. This new class of anti-retroviral molecules can recognize and cleave the integrated provirus either *in vitro* or *in vivo*, these reagents can be used for a variety of purposes for instance in research as well as in novel treatment regimes.

According to a first aspect of the present invention there is provided
10 an I-CreI variant, characterized in that at least one of the two I-CreI monomers has at least two substitutions, one in each of the two functional subdomains of the LAGLIDADG core domain situated from positions 26 to 40 and 44 to 77 of I-CreI, said variant being able to cleave a DNA target sequence from a genomically integrated provirus (GIP), and being obtainable by a method comprising at least the
15 steps of:

(a) constructing a first series of I-CreI variants having at least one substitution in a first functional subdomain of the LAGLIDADG core domain situated from positions 26 to 40 of I-CreI,

(b) constructing a second series of I-CreI variants having at least
20 one substitution in a second functional subdomain of the LAGLIDADG core domain situated from positions 44 to 77 of I-CreI,

(c) selecting and/or screening the variants from the first series of step (a) which are able to cleave a mutant I-CreI site wherein at least one of (i) the nucleotide triplet in positions -10 to -8 of the I-CreI site has been replaced with the
25 nucleotide triplet which is present in positions -10 to -8 of said DNA target sequence from the GIP and (ii) the nucleotide triplet in positions +8 to +10 has been replaced with the reverse complementary sequence of the nucleotide triplet which is present in positions -10 to -8 of said DNA target sequence from the GIP,

(d) selecting and/or screening the variants from the second series of
30 step (b) which are able to cleave a mutant I-CreI site wherein at least one of (i) the nucleotide triplet in positions -5 to -3 of the I-CreI site has been replaced with the nucleotide triplet which is present in positions -5 to -3 of said DNA target sequence

from the GIP and (ii) the nucleotide triplet in positions +3 to +5 has been replaced with the reverse complementary sequence of the nucleotide triplet which is present in position -5 to -3 of said DNA target sequence from the GIP,

(e) selecting and/or screening the variants from the first series of
5 step (a) which are able to cleave a mutant I-*CreI* site wherein at least one of (i) the nucleotide triplet in positions +8 to +10 of the I-*CreI* site has been replaced with the nucleotide triplet which is present in positions +8 to +10 of said DNA target sequence from the GIP and (ii) the nucleotide triplet in positions -10 to -8 has been replaced with the reverse complementary sequence of the nucleotide triplet which is present in
10 positions +8 to +10 of said DNA target sequence from the GIP,

(f) selecting and/or screening the variants from the second series of
step (b) which are able to cleave a mutant I-*CreI* site wherein at least one of (i) the nucleotide triplet in positions +3 to +5 of the I-*CreI* site has been replaced with the nucleotide triplet which is present in positions +3 to +5 of said DNA target sequence
15 from the GIP and (ii) the nucleotide triplet in positions -5 to -3 has been replaced with the reverse complementary sequence of the nucleotide triplet which is present in positions +3 to +5 of said DNA target sequence from the GIP,

(g) combining in a single variant, the mutation(s) in positions 26 to
40 and 44 to 77 of two variants from step (c) and step (d), to obtain a novel
20 homodimeric I-*CreI* variant which cleaves a sequence wherein (i) the nucleotide triplet in positions -10 to -8 is identical to the nucleotide triplet which is present in positions -10 to -8 of said DNA target sequence from the GIP, (ii) the nucleotide triplet in positions +8 to +10 is identical to the reverse complementary sequence of the nucleotide triplet which is present in positions -10 to -8 of said DNA target sequence
25 from the GIP, (iii) the nucleotide triplet in positions -5 to -3 is identical to the nucleotide triplet which is present in positions -5 to -3 of said DNA target sequence from the GIP and (iv) the nucleotide triplet in positions +3 to +5 is identical to the reverse complementary sequence of the nucleotide triplet which is present in positions -5 to -3 of said DNA target sequence from the GIP, and/or

(h) combining in a single variant, the mutation(s) in positions 26 to
40 and 44 to 77 of two variants from step (e) and step (f), to obtain a novel
30 homodimeric I-*CreI* variant which cleaves a sequence wherein (i) the nucleotide

triplet in positions +8 to +10 of the I-*CreI* site has been replaced with the nucleotide triplet which is present in positions +8 to +10 of said DNA target sequence from the GIP and (ii) the nucleotide triplet in positions -10 to -8 is identical to the reverse complementary sequence of the nucleotide triplet in positions +8 to +10 of said DNA target sequence from the GIP, (iii) the nucleotide triplet in positions +3 to +5 is identical to the nucleotide triplet which is present in positions +3 to +5 of said DNA target sequence from the GIP, (iv) the nucleotide triplet in positions -5 to -3 is identical to the reverse complementary sequence of the nucleotide triplet which is present in positions +3 to +5 of said DNA target sequence from the GIP,

(i) combining the variants obtained in steps (g) and (h) to form heterodimers, and

(j) selecting and/or screening the heterodimers from step (i) which are able to cleave said DNA target sequence from the GIP.

The inventors therefore provide a set of I-*CreI* variants which can recognise and cut targets in a genomically integrated provirus (GIP). Such I-*CreI* variants are potentially useful in studies into the transcriptional and regulatory behaviour of the provirus as well as potentially providing a new therapeutic route to HIV treatment by HIV provirus inactivation or alteration.

In the present Patent Application the genomically integrated provirus (GIP) refers to the DNA sequence present in one or several places in the host cell genome which was inserted following reverse transcription of the RNA virus genome and its integration into the host genome.

In the present Patent Application the terms meganuclease (s) and variant (s) and variant meganuclease (s) will be used interchangeably herein.

The inventors have therefore created a new class of meganuclease based reagents which are useful for studying a retrovirus *in vitro* and *in vivo*; this class of reagents also represent a potential new class of anti-retrovirus and in particular anti-HIV medicament, which instead of acting upon the virion or any component thereof acts upon the genomic insertion of the virus.

Targeting the integrated provirus would allow a clinician to eliminate the structure which leads to the generation of further viral particles, acting at a level that no other anti-viral therapeutic approaches have yet been developed.

Conversely, prior art therapies which act upon the different steps of the viral life cycle allow to a clinician to inhibit viral replication, but do not eliminate the source of the virions, which therefore allows for the amplification of the viral infection when the treatment is withdrawn or resistance develops.

5 The Inventors have validated this new class of anti-retrovirus reagents by generating meganuclease variants to a series of DNA targets in the genome of the HIV provirus (Figures 7, 24, 35 and 48). Four targets in the HIV provirus were chosen and the inventors set out to determine whether it was possible to generate meganucleases capable of cleaving these.

10 These target sequences are present in the LTR region, the coding sequence of the p24 protein and in the protease gene. These four targets were selected based on their therapeutic potential.

 As mentioned before, one potential therapeutic approach would be to cleavage of both LTRs of the integrated provirus which would in turn lead to excision
15 of the viral genome from the infected cells. An alternative therapeutic approach would be to targeting one or more essential genes, the p24 protein is a structural component of the viral capsid and is essential for the virus to replicate. The HIV protease, is also an essential protein that is needed for viral particle maturation, without which viral particles remain in an immature state and are not infectious. The inventors have
20 therefore established that meganuclease variants can be generated in both the sequences of essential genes as well as in regulatory non-coding sequences essential for viral replication.

 These targets were also selected based on a screen on the "Los Alamos National Laboratory" Sequence database (www.hiv.lanl.gov) to determine
25 their degree of conservation among circulating isolates, which showed a high degree of sequence conservation among the different viral strains for which the complete sequence of their genome is available.

 In the present Patent Application essential genes of the GIP provirus are those genes which must remain active in order for the GIP provirus to be
30 converted into further virions which are able to exit the host cell and infect further cells. In addition to essential genes, other types of essential genetic elements can exist such as the regulatory elements of essential genes and/or structural sequence elements

of the HIV provirus that are necessary for its packaging and/or insertion into the genome.

A combinatorial approach, as illustrated schematically in Figure 6 was used to entirely redesign the DNA binding domain of the I-CreI protein and thereby engineer novel meganucleases with fully engineered specificity.

In particular the heterodimer of step (i) may comprise monomers obtained in steps (g) and (h), with the same DNA target recognition and cleavage activity properties.

Alternatively the heterodimer of step (i) may comprise monomers obtained in steps (g) and (h), with different DNA target recognition and cleavage activity properties.

In particular the first series of I-CreI variants of step (a) are derived from a first parent meganuclease.

In particular the second series of variants of step (b) are derived from a second parent meganuclease.

In particular the first and second parent meganucleases are identical.

Alternatively the first and second parent meganucleases are different.

In particular the variant may be obtained by a method comprising the additional steps of:

(k) selecting heterodimers from step (j) and constructing a third series of variants having at least one substitution in at least one of the monomers of said selected heterodimers,

(l) combining said third series variants of step (k) and screening the resulting heterodimers for enhanced cleavage activity against said DNA target from the GIP.

The inventors have found that although specific meganucleases can be generated to a particular target in the GIP using the above method, that such meganucleases can be improved further by the additional rounds of substitution and selection against the intended target.

In particular in said step (k) the substitutions in the third series of variants are introduced by site directed mutagenesis in a DNA molecule encoding said

third series of variants, and/or by random mutagenesis in a DNA molecule encoding said third series of variants.

In the additional rounds of substitution and selection, the substitution of residues in the meganucleases can be performed randomly, that is wherein the chances of a substitution event occurring are equal chance across all the residues of the meganuclease. Or on a site directed basis wherein the chances of certain residues being subject to a substitution is higher than other residues.

In particular steps (k) and (l) are repeated at least two times and wherein the heterodimers selected in step (k) of each further iteration are selected from heterodimers screened in step (l) of the previous iteration which showed increased cleavage activity against said DNA target from the GIP.

The inventors have found that the meganucleases can be further improved by using multiple iterations of the additional steps (k) and (l).

In particular said substitution(s) in the subdomain situated from positions 26 to 40 of I-CreI are in positions 26, 28, 30, 32, 33, 38 and/or 40.

Through the inventors work they have identified the residues in the first subdomain which when altered have most effect upon altering the I-CreI enzymes specificity.

In particular said substitution(s) in the subdomain situated from positions 44 to 77 of I-CreI are in positions 44, 68, 70, 75 and/or 77.

Through the inventors work they have identified the residues in the second subdomain which when altered have most effect upon altering the I-CreI enzymes specificity.

In particular the variant comprises one or more substitutions in positions 137 to 143 of I-CreI that modify the specificity of the variant towards the nucleotide in positions ± 1 to 2, ± 6 to 7 and/or ± 11 to 12 of the target site in the GIP.

In particular the variant comprises one or more substitutions on the entire I-CreI sequence that improve the binding and/or the cleavage properties of the variant towards said DNA target sequence from the GIP.

As well as specific mutations at the residue identified above, the present invention also encompasses the substitution of any of the residues present in the I-CreI enzyme.

In particular wherein said substitutions are replacement of the initial amino acids with amino acids selected in the group consisting of A, D, E, G, H, K, N, P, Q, R, S, T, Y, C, W, L and V.

5 In particular the GIP is from the family Retroviridae and in particular from one of the genera: *Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, *Lentivirus* and *Spumavirus*.

Most particularly the GIP is selected from the group Human T-lymphotrophic virus, Human Immunodeficiency Virus.

10 Multiple examples of genomic sequences for viruses of the specified types are available from public databases such as the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) or the virus genomics and bioinformatics resources centre at University College London (http://www.biochem.ucl.ac.uk/bsm/virus_database/VIDA.html).

15 These publicly available resources together with the detailed materials and methods described in the present Patent Application mean that meganuclease variants cleaving appropriate targets in the genomes can be generated and that in turn these variants can be used to cleave the viral genomic material *in vivo* for therapeutic and/or research purposes.

In particular said DNA target sequence is from the HIV1 provirus.

20 In particular the variants may be selected from the group consisting of SEQ ID NO: 1-13; SEQ ID NO: 26-46; SEQ ID NO: 59-85; SEQ ID NO: 88-94; SEQ ID NO: 97-165; SEQ ID NO: 168-174; SEQ ID NO: 177-186; SEQ ID NO: 189-238; SEQ ID NO: 241-242; SEQ ID NO: 245-253; SEQ ID NO: 256-316.

25 In particular said DNA target is selected from the group consisting of the sequences SEQ ID NO: 319 to 342.

In particular said DNA target is within a DNA sequence essential for HIV replication, viability, packaging or virulence.

In particular the DNA target is within an essential gene or regulatory element or structural element of the HIV provirus.

30 In particular the DNA target is within the open reading frame of the HIV provirus encoding a gene or regulatory element of a gene selected from the group: GAG, POL, ENV, TAT and REV.

For example in the present Patent Application the inventors provide meganuclease variants which can cleave targets in the GAG gene (SEQ ID NO: 331, HIV1_4, Examples 16 to 23 below) and the POL gene (SEQ ID NO: 337, HIV1_5, examples 24 to 31 below) of the HIV provirus.

5 In particular the variant is a heterodimer, resulting from the association of a first and a second monomer having different mutations in positions 26 to 40 and 44 to 77 of I-*CreI*, said heterodimer being able to cleave a non-palindromic DNA target sequence from the HIV provirus.

As explained above the I-*CreI* enzyme acts as a dimer, by ensuring
10 that the variant is a heterodimer this allows a specific combination of two different I-*CreI* monomers which increases the possible targets cleaved by the variant.

In particular the heterodimeric variant is an obligate heterodimer variant having at least one pair of mutations in corresponding residues of the first and the second monomers which mediate an intermolecular interaction between the two I-
15 *CreI* monomers, wherein the first mutation of said pair(s) is in the first monomer and the second mutation of said pair(s) is in the second monomer and said pair(s) of mutations impairs the formation of functional homodimers from each monomer without preventing the formation of a functional heterodimer, able to cleave the genomic DNA target from the HIV provirus.

20 The inventors have previously established a number of residue changes which can ensure an I-*CreI* monomer is an obligate heterodimer (WO2008/093249).

In particular the monomers have at least one of the following pairs of mutations, respectively for the first and the second monomer:

25 a) the substitution of the glutamic acid in position 8 with a basic amino acid, preferably an arginine (first monomer) and the substitution of the lysine in position 7 with an acidic amino acid, preferably a glutamic acid (second monomer); the first monomer may further comprise the substitution of at least one of the lysine residues in positions 7 and 96, by an arginine,

30 b) the substitution of the glutamic acid in position 61 with a basic amino acid, preferably an arginine (first monomer) and the substitution of the lysine in position 96 with an acidic amino acid, preferably a glutamic acid (second monomer);

the first monomer may further comprise the substitution of at least one of the lysine residues in positions 7 and 96, by an arginine,

- c) the substitution of the leucine in position 97 with an aromatic amino acid, preferably a phenylalanine (first monomer) and the substitution of the phenylalanine in position 54 with a small amino acid, preferably a glycine (second monomer); the first monomer may further comprise the substitution of the phenylalanine in position 54 by a tryptophane and the second monomer may further comprise the substitution of the leucine in position 58 or lysine in position 57, by a methionine, and
- d) the substitution of the aspartic acid in position 137 with a basic amino acid, preferably an arginine (first monomer) and the substitution of the arginine in position 51 with an acidic amino acid, preferably a glutamic acid (second monomer).

In particular the variant, which is an obligate heterodimer, wherein the first and the second monomer, respectively, further comprises the D137R mutation and the R51D mutation.

In particular the variant, which is an obligate heterodimer, wherein the first monomer further comprises the K7R, E8R, E61R, K96R and L97F or K7R, E8R, F54W, E61R, K96R and L97F mutations and the second monomer further comprises the K7E, F54G, L58M and K96E or K7E, F54G, K57M and K96E mutations. According to a second aspect of the present invention there is provided a single-chain chimeric meganuclease which comprises two monomers or core domains of one or two variant(s) according to the first aspect of the present invention, or a combination of both.

An alternative approach to ensuring that the variant consists of a specific combination of monomers is to link the selected monomers for instance using a peptide linker.

In particular the single-chain meganuclease comprises a first and a second monomer according to the first aspect of the present invention, connected by a peptidic linker.

According to a third aspect of the present invention there is provided a polynucleotide fragment encoding the variant according to the first aspect of the

present invention or the single-chain chimeric meganuclease according to a second aspect of the present invention.

According to a fourth aspect of the present invention there is provided an expression vector comprising at least one polynucleotide fragment
5 according to the third aspect of the present invention.

In particular the expression vector, includes a targeting construct comprising a sequence to be introduced flanked by sequences sharing homologies with the regions surrounding said DNA target sequence from the provirus.

One important use of a variant according to the present invention is
10 in increasing the incidence of homologous recombination events at or around the site where the variant cleaves its target. The present invention therefore also relates to a unified genetic construct which encodes the variant under the control of suitable regulatory sequences as well as sequences homologous to portions of the provirus surrounding the variant DNA target site. Following cleavage of the target site by the
15 variant these homologous portions can act as a complementary sequences in a homologous recombination reactions with the provirus replacing the existing provirus sequence with a new sequence engineered between the two homologous portions in the unified genetic construct.

Preferably, homologous sequences of at least 50 bp, preferably more
20 than 100 bp and more preferably more than 200 bp are used. Shared DNA homologies are located in regions flanking upstream and downstream the site of the break and the DNA sequence to be introduced should be located between the two arms.

Therefore, the targeting construct is preferably from 200 bp to 6000 bp, more preferably from 1000 bp to 2000 bp; it comprises: a sequence which has at
25 least 200 bp of homologous sequence flanking the target site, for repairing the cleavage and a sequence for inactivating the provirus and/or a sequence of an exogenous gene of interest which it is intended to insert at the site of the DNA repair event by homologous recombination.

For the insertion of a sequence, DNA homologies are generally
30 located in regions directly upstream and downstream to the site of the break (sequences immediately adjacent to the break; minimal repair matrix). However, when the insertion is associated with a deletion of ORF sequences flanking the cleavage site,

shared DNA homologies are located in regions upstream and downstream the region of the deletion.

A vector which can be used in the present invention includes, but is not limited to, a viral vector, a plasmid, a RNA vector or a linear or circular DNA or
5 RNA molecule which may consists of a chromosomal, non chromosomal, semi-synthetic or synthetic nucleic acids. Preferred vectors are those capable of autonomous replication (episomal vector) and/or expression of nucleic acids to which they are linked (expression vectors). Large numbers of suitable vectors are known to those of skill in the art and commercially available.

10 Viral vectors include retrovirus, adenovirus, parvovirus (e.g. adeno associated viruses), coronavirus, negative strand RNA viruses such as orthomyxovirus (e. g., influenza virus), rhabdovirus (e. g., rabies and vesicular stomatitis virus), paramyxovirus (e. g. measles and Sendai), positive strand RNA viruses such as picornavirus and alphavirus, and double-stranded DNA viruses including adenovirus,
15 herpesvirus (e. g., Herpes Simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus), and poxvirus (e. g., vaccinia, fowlpox and canarypox). Other viruses include Norwalk virus, togavirus, flavivirus, reoviruses, papovavirus, hepadnavirus, and hepatitis virus, for example. Examples of retroviruses include: avian leukosis-sarcoma, mammalian C-type, B-type viruses, D type viruses, HTLV-
20 BLV group, lentivirus, spumavirus (Coffin, J. M., Retroviridae: The viruses and their replication, In Fundamental Virology, Third Edition, B. N. Fields, et al., Eds., Lippincott-Raven Publishers, Philadelphia, 1996).

Vectors can comprise selectable markers, for example: neomycin phosphotransferase, histidinol dehydrogenase, dihydrofolate reductase, hygromycin
25 phosphotransferase, herpes simplex virus thymidine kinase, adenosine deaminase, glutamine synthetase, and hypoxanthine-guanine phosphoribosyl transferase (HRPT) for eukaryotic cell culture; TRP1 for *S. cerevisiae*; tetracycline, rifampicin or ampicillin resistance in *E. coli*.

In particular for the purposes of gene therapy and in accordance with
30 a preferred embodiment of the present invention, the viral vector is selected from the group comprising lentiviruses, Adeno-associated viruses (AAV) and Adenoviruses.

In accordance with another aspect of the present invention the variant and targeting construct may be on different nucleic acid constructs.

In accordance with another aspect of the present invention the variant in the form of a peptide and the targeting construct as a nucleic acid molecule
5 may be used in combination.

In particular, wherein said sequence to be introduced is a sequence which inactivates the HIV provirus.

In particular, wherein the sequence which inactivates the HIV provirus comprises in the 5' to 3' orientation: a first transcription termination
10 sequence and a marker cassette including a promoter, the marker open reading frame and a second transcription termination sequence, and said sequence interrupts the transcription of the coding sequence.

In particular, wherein said sequence sharing homologies with the regions surrounding DNA target sequence is from the HIV provirus or a fragment of
15 the HIV provirus comprising sequences upstream and downstream of the cleavage site, so as to allow the deletion of coding sequences flanking the cleavage site.

According to a fifth aspect of the present invention there is provided a host cell which is modified by a polynucleotide according to a third aspect of the present invention or a vector according to a fourth aspect of the present invention.

20 A cell according to the present invention may be made according to a method, comprising at least the step of:

(a) introducing into a cell, a meganuclease, as defined above, so as to induce a double stranded cleavage at a site of interest of the GIP comprising a DNA recognition and cleavage site of said meganuclease, and thereby generate a
25 genomically modified cell having repaired the double-strands break, by non-homologous end joining, and

(b) isolating the genomically modified cell of step (a), by any appropriate mean.

The cell which is modified may be any cell of interest. For making
30 transgenic/knock-out animals, the cells are pluripotent precursor cells such as embryo-derived stem (ES) cells, which are well-known in the art. For making recombinant cell lines, the cells may advantageously be human cells, for example PerC6 (Fallaux et al.,

Hum. Gene Ther. 9, 1909-1917, 1998) or HEK293 (ATCC # CRL-1573) cells or an immortal T lymphocyte line such as Jurkat (Schneider et al (1977). *Int J Cancer* 19 (5): 621-6.). The meganuclease can be provided directly to the cell or through an expression vector comprising the polynucleotide sequence encoding said
5 meganuclease linked to regulatory sequences suitable for directing its expression in the cell used.

Such a modified cell line would have a number of potential uses including the elucidation of aspects of the biology of the modified GIP as well as a model for screening compounds and other substances for therapeutic effects against
10 cells comprising the modified GIP.

According to a sixth aspect of the present invention there is provided a non-human transgenic animal which is modified by a polynucleotide according to a third aspect of the present invention or a vector according to a fourth aspect of the present invention.

15 The subject-matter of the present invention is also a method for making an animal which comprises a modified GIP, comprising at least the step of:

(a) introducing into a pluripotent precursor cell or an embryo of an animal, a meganuclease, as defined above, so as to induce a double stranded cleavage at a site of interest of the GIP comprising a DNA recognition and cleavage site of said
20 meganuclease, and thereby generate a genomically modified precursor cell or embryo having repaired the double-strands break by non-homologous end joining,

(b) developing the genomically modified animal precursor cell or embryo of step (a) into a chimeric animal, and

(c) deriving a transgenic animal from a chimeric animal of step (b).

25 Alternatively, the GIP may be inactivated by insertion of a sequence of interest by homologous recombination between the genome of the animal and a targeting DNA construct according to the present invention.

In particular the targeting DNA is introduced into the cell under conditions appropriate for introduction of the targeting DNA into the site of interest.

30 In particular, step (b) comprises the introduction of the genomically modified precursor cell obtained in step (a), into blastocysts, so as to generate chimeric animals.

Such a transgenic animal could be used as a multicellular animal model to elucidate aspects of the biology of the GIP, by means of engineering the provirus present in the progenitor cell line. Such transgenic animals also could be used to screen and characterise the effects of for instance novel anti-HIV medicaments.

5 In particular the targeting DNA construct is inserted in a vector.

For making transgenic animals/recombinant cell lines, including human cell lines expressing an heterologous protein of interest, the targeting DNA comprises the sequence of the exogenous gene encoding the protein of interest, and eventually a marker gene, flanked by sequences upstream and downstream of and
10 essential gene in the HIV provirus, as defined above, so as to generate genomically modified cells (animal precursor cell or embryo/animal or human cell) having replaced the HIV gene by the exogenous gene of interest, by homologous recombination.

The exogenous gene and the marker gene are inserted in an appropriate
15 expression cassette, as defined above, in order to allow expression of the heterologous protein/marker in the transgenic animal/recombinant cell line.

The meganuclease can be used either as a polypeptide or as a polynucleotide construct encoding said polypeptide. It is introduced into somatic cells of an individual, by any convenient means well-known to those in the art, which are
20 appropriate for the particular cell type, alone or in association with either at least an appropriate vehicle or carrier and/or with the targeting DNA.

According to the present invention, the meganuclease (polypeptide) is associated with:

- liposomes, polyethyleneimine (PEI); in such a case said association
25 is administered and therefore introduced into somatic target cells.

- membrane translocating peptides (Bonetta, *The Scientist*, 2002, 16, 38; Ford et al., *Gene Ther.*, 2001, 8, 1-4 ; Wadia and Dowdy, *Curr. Opin. Biotechnol.*, 2002, 13, 52-56); in such a case, the sequence of the variant/single-chain meganuclease is fused with the sequence of a membrane translocating peptide (fusion
30 protein).

Alternatively, the meganuclease (polynucleotide encoding said meganuclease) and/or the targeting DNA is inserted in a vector. Vectors comprising

targeting DNA and/or nucleic acid encoding a meganuclease can be introduced into a cell by a variety of methods (e.g., injection, direct uptake, projectile bombardment, liposomes, electroporation). Meganucleases can be stably or transiently expressed into cells using expression vectors. Techniques of expression in eukaryotic cells are well known to those in the art. (See Current Protocols in Human Genetics: Chapter 12 “Vectors For Gene Therapy” & Chapter 13 “Delivery Systems for Gene Therapy”). Optionally, it may be preferable to incorporate a nuclear localization signal into the recombinant protein to be sure that it is expressed within the nucleus.

Once in a cell, the meganuclease and if present, the vector comprising targeting DNA and/or nucleic acid encoding a meganuclease are imported or translocated by the cell from the cytoplasm to the site of action in the nucleus.

According to a seventh aspect of the present invention there is provided a transgenic plant which is modified by a polynucleotide according to a third aspect of the present invention or a vector according to a fourth aspect of the present invention.

According to a further aspect of the present invention there is provided the use of at least one variant according to the first aspect of the present invention, or at least one single-chain chimeric meganuclease according to the second aspect of the present invention, or at least one vector according to the fourth aspect of the present invention, for genome engineering, for therapeutic or non-therapeutic purposes.

In particular the variant or single-chain chimeric meganuclease or vector is associated with a targeting DNA construct.

In particular the use of the variant is for inducing a double-strand break in a site of interest of the GIP comprising a genomic DNA target sequence, thereby inducing a DNA recombination event, a DNA loss or cell death.

According to the invention, said double-strand break is for: modifying a specific sequence in the GIP, so as to induce restoration of a GIP function such as replication, or to attenuate or activate the GIP or a gene therein, introducing a mutation into a site of interest of a GIP gene, introducing an exogenous gene or a part thereof, inactivating or deleting the GIP or a part thereof or leaving the DNA unrepaired and degraded.

In particular this present aspect of the present invention relates to the use of a meganuclease variant to treat HIV infection, by inactivating the HIV provirus by therapeutic genome engineering.

According to this aspect of the present invention the use of the
5 meganuclease according to the present invention, comprises at least the following steps:

1) introducing a double-strand break at least one site of interest in the HIV provirus comprising at least one recognition and cleavage site of said meganuclease, by contacting said cleavage site with said meganuclease ;

10 2) providing a targeting DNA construct comprising the sequence to be introduced flanked by sequences sharing homologies to the targeted locus (i). Wherein the meganuclease is be provided directly to the cell or through an expression vector comprising the polynucleotide sequence encoding of the meganuclease and is suitable for its expression in the host cell.

15 This strategy is used to introduce a DNA sequence at the target site, for example to generate a HIV provirus knock-in or knock-out animal model or cell lines that can be used for drug testing or in the case of a cell line, which can be used for administration into a patient from whom it was derived.

According to a further aspect of the present invention the use of the
20 meganuclease, comprises at least the following steps:

1) introducing a double-strand break at a site of interest of the HIV provirus comprising at least one recognition and cleavage site of said meganuclease, by contacting said cleavage site with said meganuclease;

25 2) maintaining said broken genomic locus under conditions appropriate for homologous recombination with chromosomal DNA sharing homologies to regions surrounding the cleavage site.

As well as inactivating the provirus using a targeting construct, a significant number of inter chromosome arm recombination events are also expected to occur following cleavage of the provirus target. The recombination of chromosome
30 arms occurs most frequently during mitosis, but can also occur as part of the repair mechanism for DNA strand breaks. Such an inter chromosome arm recombination event would either lead to the elimination of the non homologous portions on either

side of the break (e.g. the provirus) or more likely cause portions of the provirus to be recombined onto different chromosome arms. In either event this would lead to the inactivation of the provirus.

According to still further aspect of the present invention the use of
5 the meganuclease, comprises at least the following steps:

- 1) introducing a double-strand break at a site of interest of the HIV provirus comprising at least one recognition and cleavage site of said meganuclease, by contacting said cleavage site with said meganuclease ;
- 2) maintaining said broken genomic locus under conditions appropriate for repair of the double-strands break by non-homologous end joining.
10

According to a further aspect of the present invention the variant is used for genome therapy to knock-out in animals/cells the GIP, in particular a sequence is introduced which inactivates the HIV provirus.

All HIV proviruses present in the cell have to be targeted in order to
15 totally inactivate the pathogenicity of the virus. In addition, the introduced sequence may also delete the HIV provirus or part thereof, and introduce an exogenous gene or part thereof (knock-in/gene replacement). For making knock-in animals/cells the DNA which repairs the site of interest may comprise the sequence of an exogenous gene of interest, and a selection marker, such as the G418 resistance gene. Alternatively, the
20 sequence to be introduced can be any other sequence used to alter the chromosomal DNA in some specific way including a sequence used to modify a specific sequence, to attenuate or activate the endogenous gene of interest in the HIV provirus or to introduce a mutation into a site of interest in the HIV provirus. Such chromosomal DNA alterations may be used for genome engineering (animal models and recombi-
25 nant cell lines including human cell lines).

Inactivation of the HIV provirus may occur by insertion of a transcription termination signal that will interrupt the transcription of an essential gene such as GAG, POL and ENV and result in a truncated protein. In this case, the sequence to be introduced comprises, in the 5' to 3' orientation: at least a transcription
30 termination sequence (polyA1), preferably said sequence further comprises a marker cassette including a promoter and the marker open reading frame (ORF) and a second transcription termination sequence for the marker gene ORF (polyA2). This strategy

can be used with any variant cleaving a target downstream of the relevant gene promoter and upstream of the stop codon.

Inactivation of the HIV provirus may also occur by insertion of a marker gene within an essential gene of HIV, which would disrupt the coding sequence. The insertion can in addition be associated with deletions of ORF sequences flanking the cleavage site and eventually, the insertion of an exogenous gene of interest (gene replacement).

In addition, inactivation of the HIV provirus may also occur by insertion of a sequence that would destabilize the mRNA transcript of an essential gene.

The present invention also provides a composition characterized in that it comprises at least one variant as defined above (variant or single-chain derived chimeric meganuclease) and/or at least one expression vector encoding the variant, as defined above.

In particular the composition comprises more than one variant, wherein each of the variants is directed towards a different target sequence in the provirus.

In particular the composition comprises a targeting DNA construct comprising a sequence which inactivates the HIV provirus, flanked by sequences sharing homologies with the genomic DNA cleavage site of said variant, as defined above.

Preferably, said targeting DNA construct is either included in a recombinant vector or it is included in an expression vector comprising the polynucleotide(s) encoding the variant according to the invention.

The subject-matter of the present invention is also the use of at least one meganuclease and/or one expression vector, as defined above, for the preparation of a medicament for preventing, improving or curing HIV infection in an individual in need thereof.

The subject-matter of the present invention is also the use of at least one variant and/or one expression vector, as defined above, for the preparation of a medicament for preventing, improving or curing a pathological condition associated with HIV infection in an individual in need thereof.

The use of the meganuclease may comprise at least the step of (a) inducing in somatic tissue(s) of the donor/ individual a double stranded cleavage at a site of interest of the HIV provirus comprising at least one recognition and cleavage site of said meganuclease by contacting said cleavage site with said meganuclease, and (b) introducing into said somatic tissue(s) a targeting DNA, wherein said targeting DNA comprises (1) DNA sharing homologies to the region surrounding the cleavage site and (2) DNA which inactivates the HIV provirus upon recombination between the targeting DNA and the chromosomal DNA, as defined above. The targeting DNA is introduced into the somatic tissues(s) under conditions appropriate for introduction of the targeting DNA into the site of interest. The targeting construct may comprise sequences for deleting the HIV provirus or a portion thereof and introducing the sequence of an exogenous gene of interest (gene replacement).

In this case the use of the meganuclease comprises at least the step of: inducing in somatic tissue(s) of the donor/individual a double stranded cleavage at a site of interest of the HIV provirus comprising at least one recognition and cleavage site of the meganuclease by contacting the cleavage site with the meganuclease, and thereby inducing mutagenesis of an open reading frame in the HIV provirus by repair of the double-strands break by non-homologous end joining.

According to the present invention, said double-stranded cleavage may be induced, *ex vivo* by introduction of said meganuclease into infected cells isolated for instance from the circulatory system of the donor/individual and then transplantation of the modified cells back into the diseased individual.

The subject-matter of the present invention is also a method for preventing, improving or curing HIV infection, in an individual in need thereof, said method comprising at least the step of administering to said individual a composition as defined above, by any means.

For purposes of therapy, the meganucleases and a pharmaceutically acceptable excipient are administered in a therapeutically effective amount. Such a combination is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of the recipient. In the present context, an agent is physiologically significant if its presence

results in a decrease in the severity of one or more symptoms of the targeted HIV infection.

In particular as far as possible the meganuclease comprising compositions should be non-immunogenic, i.e., engender little or no adverse immunological response. A variety of methods for ameliorating or eliminating deleterious immunological reactions of this sort can be used in accordance with the invention. One means of achieving this is to ensure that the meganuclease is substantially free of N-formyl methionine. Another way to avoid unwanted immunological reactions is to conjugate meganucleases to polyethylene glycol ("PEG") or polypropylene glycol ("PPG") (preferably of 500 to 20,000 daltons average molecular weight (MW)). Conjugation with PEG or PPG, as described by Davis et al. (US 4,179,337) for example, can provide non-immunogenic, physiologically active, water soluble endonuclease conjugates with anti-viral activity. Similar methods also using a polyethylene--polypropylene glycol copolymer are described in Saifer et al. (US 5,006,333).

In accordance with a further aspect of the present invention, the invention also relates to meganuclease variants, related materials and uses thereof which recognize non-virus retroelements and/or the integrated genomes of viruses which do not have mechanisms to integrate into the host cell genome.

Non-virus retroelements are endogenous genomic DNA elements that include the gene for reverse transcriptase and are also known as class I transposable elements. These retrotransposons, include the long terminal repeat (LTR) retrotransposons, non-LTR retroposons and group II mitochondrial introns. They are thought to be derived from partially inactivated retroviruses which have lost the ability to form infective virus particles. These genetic elements however are increasingly becoming associated with various diseases, in particular cancers and immune disorders which result from the integration of the element into a site close to a gene (s) whose misregulation leads to the observed disease phenotype.

The present invention therefore also relates to meganuclease variants which can be used to cleave a genomic retrotransposon either in a specific tissue or cell type or more generally so as to treat the disease phenotype using one or more of the mechanisms described above.

The present invention also relates to meganuclease variants which can recognise and cleave targets in genomic insertions of viruses which do not normally insert into the host cell genome. The non-specific insertion of viral genetic material into the host cell genome is a disease causing mechanism which is currently
5 being investigated. For example in the important virus hepatitis B, chronic infection with this virus is associated with a greatly elevated risk of hepatocellular carcinoma. In the past this association has been explained as a side effect of the episomal hepatitis B genome upon the hepatocyte host cells. Although this is doubtless true, recently the random genomic insertion of copies of the hepatitis B genome into the host cell
10 genome has also been shown to be a causative factor in hepatocyte carcinoma (Goodarzi et al., 2008, Hep. Mon; 8 (2): 129-133).

Hepatocellular carcinoma is one of the most common cancers in the world and hence a treatment for this condition, using a meganuclease variant which can cleave the randomly integrated hepatitis B genome and have a therapeutic affect
15 upon hepatocytes via one or more of mechanisms detailed above is therefore also within the scope of the present invention as are more generally meganuclease variants to genomically integrated copies of virus genetic material which cause a disease phenotype.

Definitions

20 Throughout the present Patent Application a number of terms and features are used to present and describe the present invention, to clarify the meaning of these terms a number of definitions are set out below and wherein a feature or term is not otherwise specifically defined or obvious from its context the following definitions apply.

25 - Amino acid residues in a polypeptide sequence are designated herein according to the one-letter code, in which, for example, Q means Gln or Glutamine residue, R means Arg or Arginine residue and D means Asp or Aspartic acid residue.

- Amino acid substitution means the replacement of one amino acid
30 residue with another, for instance the replacement of an Arginine residue with a Glutamine residue in a peptide sequence is an amino acid substitution.

- Altered/enhanced/increased cleavage activity, refers to an increase in the detected level of meganuclease cleavage activity (see below) against a target DNA sequence by a first meganuclease in comparison to the activity of a second meganuclease against the target DNA sequence. Normally the first meganuclease will be a variant of the second and comprise one or more substituted amino acid residues in comparison to the second meganuclease.

- by "beta-hairpin" it is intended two consecutive beta-strands of the antiparallel beta-sheet of a LAGLIDADG homing endonuclease core domain ($\beta_1\beta_2$ or $\beta_3\beta_4$) which are connected by a loop or a turn,

- by "chimeric DNA target" or "hybrid DNA target" it is intended the fusion of a different half of two parent meganuclease target sequences. In addition at least one half of said target may comprise the combination of nucleotides which are bound by at least two separate subdomains (combined DNA target).

- Cleavage activity: the cleavage activity of the variant according to the invention may be measured by any well-known, *in vitro* or *in vivo* cleavage assay, such as those described in the International PCT Application WO 2004/067736; Epinat *et al.*, Nucleic Acids Res., 2003, 31, 2952-2962; Chames *et al.*, Nucleic Acids Res., 2005, 33, e178; Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458, and Arnould *et al.*, J. Mol. Biol., 2007, 371, 49-65. For example, the cleavage activity of the variant of the invention may be measured by a direct repeat recombination assay, in yeast or mammalian cells, using a reporter vector. The reporter vector comprises two truncated, non-functional copies of a reporter gene (direct repeats) and the genomic (non-palindromic) DNA target sequence within the intervening sequence, cloned in a yeast or a mammalian expression vector. Usually, the genomic DNA target sequence comprises one different half of each (palindromic or pseudo-palindromic) parent homodimeric meganuclease target sequence. Expression of the heterodimeric variant results in a functional endonuclease which is able to cleave the genomic DNA target sequence. This cleavage induces homologous recombination between the direct repeats, resulting in a functional reporter gene (LacZ, for example), whose expression can be monitored by an appropriate assay. The specificity of the cleavage by the variant may be assessed by comparing the cleavage of the (non-palindromic) DNA

target sequence with that of the two palindromic sequences cleaved by the parent homodimeric meganucleases or compared with wild type meganuclease.

- by "selection or selecting" it is intended to mean the isolation of one or more meganuclease variants based upon an observed specified phenotype, for instance altered cleavage activity. This selection can be of the variant in a peptide form upon which the observation is made or alternatively the selection can be of a nucleotide coding for selected meganuclease variant.

- by "screening" it is intended to mean the sequential or simultaneous selection of one or more meganuclease variant (s) which exhibits a specified phenotype such as altered cleavage activity.

- by "derived from" it is intended to mean a meganuclease variant which is created from a parent meganuclease and hence the peptide sequence of the meganuclease variant is related to (primary sequence level) but derived from (mutations) the sequence peptide sequence of the parent meganuclease.

- by "domain" or "core domain" it is intended the "LAGLIDADG homing endonuclease core domain" which is the characteristic $\alpha_1\beta_1\beta_2\alpha_2\beta_3\beta_4\alpha_3$ fold of the homing endonucleases of the LAGLIDADG family, corresponding to a sequence of about one hundred amino acid residues. Said domain comprises four beta-strands ($\beta_1\beta_2\beta_3\beta_4$) folded in an antiparallel beta-sheet which interacts with one half of the DNA target. This domain is able to associate with another LAGLIDADG homing endonuclease core domain which interacts with the other half of the DNA target to form a functional endonuclease able to cleave said DNA target. For example, in the case of the dimeric homing endonuclease I-CreI (163 amino acids), the LAGLIDADG homing endonuclease core domain corresponds to the residues 6 to 94.

- by "DNA target", "DNA target sequence", "target sequence", "target-site", "target", "site"; "site of interest"; "recognition site", "recognition sequence", "homing recognition site", "homing site", "cleavage site" it is intended a 20 to 24 bp double-stranded palindromic, partially palindromic (pseudo-palindromic) or non-palindromic polynucleotide sequence that is recognized and cleaved by a LAGLIDADG homing endonuclease such as I-CreI, or a variant, or a single-chain chimeric meganuclease derived from I-CreI. These terms refer to a distinct DNA location, preferably a genomic location, at which a double stranded break (cleavage) is

to be induced by the meganuclease. The DNA target is defined by the 5' to 3' sequence of one strand of the double-stranded polynucleotide, as indicated for C1221 (see figure 1). Cleavage of the DNA target occurs at the nucleotides at positions +2 and -2, respectively for the sense and the antisense strand. Unless otherwise indicated,
5 the position at which cleavage of the DNA target by an I-Cre I meganuclease variant occurs, corresponds to the cleavage site on the sense strand of the DNA target.

- by "DNA target half-site", "half cleavage site" or half-site" it is intended the portion of the DNA target which is bound by each LAGLIDADG homing endonuclease core domain.

10 - by "DNA target sequence from the HIV provirus" it is intended a 20 to 24 bp sequence of the HIV provirus which is recognized and cleaved by a meganuclease variant. In particular the DNA target sequence from then HIV provirus is in an essential gene sequence and/or within an essential regulatory sequence and/or within an essential structural sequence of the HIV provirus.

15 - by "first/second/third/nth series of variants" it is intended a collection of variant meganucleases, each of which comprises one or more amino acid substitution in comparison to a parent meganuclease from which all the variants in the series are derived.

- by "functional variant" it is intended a variant which is able to
20 cleave a DNA target sequence, preferably said target is a new target which is not cleaved by the parent meganuclease. For example, such variants have amino acid variation at positions contacting the DNA target sequence or interacting directly or indirectly with said DNA target.

- by "heterodimer" it is intended to mean a meganuclease comprising
25 two non-identical monomers. In particular the monomers may differ from each other in their peptide sequence and/or in the DNA target half-site which they recognise and cleave.

- by "homologous" is intended a sequence with enough identity to
30 another one to lead to a homologous recombination between sequences, more particularly having at least 95 % identity, preferably 97 % identity and more preferably 99 %.

- by "I-CreI" it is intended the wild-type I-CreI having the sequence of pdb accession code 1g9y, corresponding to the sequence SEQ ID NO: 344 in the sequence listing.

- by "I-CreI variant with novel specificity" it is intended a variant
 5 having a pattern of cleaved targets different from that of the parent meganuclease. The terms "novel specificity", "modified specificity", "novel cleavage specificity", "novel substrate specificity" which are equivalent and used indifferently, refer to the specificity of the variant towards the nucleotides of the DNA target sequence. In the present Patent Application all the I-CreI variants described comprise an additional
 10 Alanine after the first Methionine of the wild type I-CreI sequence (SEQ ID NO: 344). These variants also comprise two additional Alanine residues and an Aspartic Acid residue after the final Proline of the wild type I-CreI sequence. These additional residues do not affect the properties of the enzyme and to avoid confusion these additional residues do not affect the numeration of the residues in I-CreI or a variant
 15 referred in the present Patent Application, as these references exclusively refer to residues of the wild type I-CreI enzyme (SEQ ID NO: 344) as present in the variant, so for instance residue 2 of I-CreI is in fact residue 3 of a variant which comprises an additional Alanine after the first Methionine.

- by "I-CreI site" it is intended a 22 to 24 bp double-stranded DNA
 20 sequence which is cleaved by I-CreI. I-CreI sites include the wild-type (natural) non-palindromic I-CreI homing site and the derived palindromic sequences such as the sequence 5'- t₁₂c₋₁₁a₋₁₀a₋₉a₋₈a₋₇c₋₆g₋₅t₋₄c₋₃g₋₂t₋₁a₊₁c₊₂g₊₃a₊₄c₊₅g₊₆t₊₇t₊₈t₊₉t₊₁₀g₊₁₁a₊₁₂ (SEQ ID NO: 343), also called C1221 .

- "identity" refers to sequence identity between two nucleic acid
 25 molecules or polypeptides. Identity can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base, then the molecules are identical at that position. A degree of similarity or identity between nucleic acid or amino acid sequences is a function of the number of identical or matching nucleotides at positions
 30 shared by the nucleic acid sequences. Various alignment algorithms and/or programs may be used to calculate the identity between two sequences, including FASTA, or

BLAST which are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, e.g., default settings.

- by "meganuclease", it is intended an endonuclease having a double-stranded DNA target sequence of 12 to 45 bp. The meganuclease is either a dimeric enzyme, wherein each domain is on a monomer or a monomeric enzyme comprising the two domains on a single polypeptide.

- by "meganuclease domain", it is intended the region which interacts with one half of the DNA target of a meganuclease and is able to associate with the other domain of the same meganuclease which interacts with the other half of the DNA target to form a functional meganuclease able to cleave said DNA target.

- by "meganuclease variant" or "variant" it is intended a meganuclease obtained by replacement of at least one residue in the amino acid sequence of the parent meganuclease (natural or variant meganuclease) with a different amino acid.

- by "monomer" it is intended to mean a peptide encoded by the open reading frame of the *I-CreI* gene or a variant thereof, which when allowed to dimerise forms a functional *I-CreI* enzyme. In particular the monomers dimerise via interactions mediated by the LAGLIDADG motif.

- by "mutation" is intended the substitution, deletion, insertion of one or more nucleotides/amino acids in a polynucleotide (cDNA, gene) or a polypeptide sequence. Said mutation can affect the coding sequence of a gene or its regulatory sequence. It may also affect the structure of the genomic sequence or the structure/stability of the encoded mRNA.

- Nucleotides are designated as follows: one-letter code is used for designating the base of a nucleoside: a is adenine, t is thymine, c is cytosine, and g is guanine. For the degenerated nucleotides, r represents g or a (purine nucleotides), k represents g or t, s represents g or c, w represents a or t, m represents a or c, y represents t or c (pyrimidine nucleotides), d represents g, a or t, v represents g, a or c, b represents g, t or c, h represents a, t or c, and n represents g, a, t or c.

- by "parent meganuclease" it is intended to mean a wild type meganuclease or a variant of such a wild type meganuclease with identical properties or alternatively a meganuclease with some altered characteristic in comparison to a

wild type version of the same meganuclease. In the present invention the parent meganuclease can refer to the initial meganuclease from which the first series of variants are derived in step a. or the meganuclease from which the second series of variants are derived in step b., or the meganuclease from which the third series of variants are derived in step k.

- by "peptide linker" it is intended to mean a peptide sequence of at least 10 and preferably at least 17 amino acids which links the C terminal amino acid residue of the first monomer to the N terminal residue of the second monomer and which allows the two variant monomers to adopt the correct conformation for activity and which does not alter the specificity of either of the monomers for their targets.

- by "subdomain" it is intended the region of a LAGLIDADG homing endonuclease core domain which interacts with a distinct part of a homing endonuclease DNA target half-site.

- by "single-chain meganuclease", "single-chain chimeric meganuclease", "single-chain meganuclease derivative", "single-chain chimeric meganuclease derivative" or "single-chain derivative" it is intended a meganuclease comprising two LAGLIDADG homing endonuclease domains or core domains linked by a peptidic spacer. The single-chain meganuclease is able to cleave a chimeric DNA target sequence comprising one different half of each parent meganuclease target sequence.

- by "targeting DNA construct/minimal repair matrix/repair matrix" it is intended to mean a DNA construct comprising a first and second portions which are homologous to regions 5' and 3' of the DNA target *in situ*. The DNA construct also comprises a third portion positioned between the first and second portion which comprise some homology with the corresponding DNA sequence *in situ* or alternatively comprise no homology with the regions 5' and 3' of the DNA target *in situ*. Following cleavage of the DNA target, a homologous recombination event is stimulated between the genome containing the HIV provirus and the repair matrix, wherein the genomic sequence containing the DNA target is replaced by the third portion of the repair matrix and a variable part of the first and second portions of the repair matrix.

- by "vector" is intended a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked into a host cell *in vitro*, *in*

vivo or *ex vivo*. For a better understanding of the invention and to show how the same may be carried into effect, there will now be shown by way of example only, specific embodiments, methods and processes according to the present invention with reference to the accompanying drawings in which:

5 Figure 1: Schematic representation of an HIV-1 viral particle. The two molecules of genomic RNA are represented, together with the RT, inside the viral capsid. The envelope, derived from the membrane of the infected cells, contains the envelope glycoproteins gp41 and gp120.

 Figure 2: **A:** Organization of the HIV-1 genomic RNA molecules. Different genes are represented with different shades of grey, and the proteins encoded by these genes are represented in the lower part of the panel. **B:** Genetic organization of the integrated HIV-1 provirus, showing the structure of the LTRs after duplication of the U3 and U5 sequences during reverse transcription.

 Figure 3: Tridimensional structure of the I-CreI homing endonuclease bound to its DNA target. The catalytic core is surrounded by two $\alpha\beta\alpha\beta\alpha$ folds forming a saddle-shaped interaction interface above the DNA major groove.

 Figure 4: Different I-CreI variants binding different sequences derived from the I-CreI target sequence (top right and bottom left) to obtain heterodimers or single chain fusion molecules cleaving non palindromic chimeric targets (bottom right).

 Figure 5: Shows a schematic representation of the smaller independent subunits of the I-CreI meganuclease, i.e., subunit within a single monomer or $\alpha\beta\alpha\beta\alpha$ fold (top right and bottom left). These independent subunits allow for the design of novel chimeric molecules (bottom right), by combination of mutations within a same monomer. Such molecules would cleave palindromic chimeric targets (bottom right).

 Figure 6: Shows a schematic representation of a method to combine four different subdomains so as to generate a custom meganuclease which cleaves a selected target.

 Figure 7: The HIV1_1 target sequence (SEQ ID NO:319) and its derivatives. In the HIV1_1.2 target (SEQ ID NO:320), the ACAC sequence in the

middle of the target is replaced with GTAC, the bases found in C1221 (SEQ ID NO:343). HIV1_1.3 (SEQ ID NO:321) is the palindromic sequence derived from the left part of HIV1_1.2, (SEQ ID NO:320) and HIV1_1.4 (SEQ ID NO:322) is the palindromic sequence derived from the right part of HIV1_1.2 (SEQ ID NO:320).
 5 HIV1_1.5 (SEQ ID NO:323) and HIV1_1.6 (SEQ ID NO:324) are pseudo-palindromic targets derived, respectively, from HIV1_1.3 (SEQ ID NO:321) and HIV1_1.4 (SEQ ID NO:322), containing the natural ACAC sequence in the middle of the target. As shown in the Figure, the boxed motives from 10AGA_P, 10TGG_P, 5TAC_P and 5CTG_P are found in the HIV1_1 series of targets.

10 Figure 8: pCLS1055 plasmid map.

Figure 9: pCLS0542 plasmid map.

Figure 10: Cleavage of HIV1_1.3 (SEQ ID NO:321) target by combinatorial variants. The figure displays an example of screening of I-CreI combinatorial variants with the HIV1_1.3 target (SEQ ID NO:321). On the filter, the
 15 positive variants correspond to: B10, SEQ ID NO:1; C1, SEQ ID NO:2; C7, SEQ ID NO:3; C10, SEQ ID NO:4; C3, SEQ ID NO:5; all described in Table II. Each cluster contains 4 spots. On the two spots on the left, a yeast strain harboring the HIV1_1.3 target (SEQ ID NO:321) has been mated with another yeast strain containing the meganuclease variants. The two spots on the right contain the same negative or
 20 positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3).

Figure 11: pCLS1107 plasmid map.

Figure 12: Cleavage of HIV1_1.4 (SEQ ID NO:322) and HIV1_1.6
 25 (SEQ ID NO:324) targets by combinatorial variants. The figure displays an example of screening of I-CreI combinatorial variants with the HIV1_1.4 (SEQ ID NO:322) and HIV1_1.6 (SEQ ID NO:324) targets. On the filter, the positive variants correspond to: C8, SEQ ID NO:7; A5, SEQ ID NO:8; A1, SEQ ID NO:9; A12, SEQ ID NO:10; C3, SEQ ID NO:11; all described in Table IV. Each cluster contains 4
 30 spots. On the two spots on the left, a yeast strain harboring the HIV1_1.4 or the HIV1_1.6 targets have been mated with another yeast strain containing the meganuclease variants. The two spots on the right contain the same negative or

positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3)

Figure 13: Cleavage of the HIV1_1.2 (SEQ ID NO:320) and HIV1_1 (SEQ ID NO:319) target sequences by heterodimeric combinatorial variants. Left panel: Example of screening of combinations of I-CreI variants against the HIV1_1.2 target. Right panel: Screening of the same combinations of I-CreI variants against the HIV1_1 target. Some heterodimers resulted in cleavage of the HIV1_1.2 target (SEQ ID NO:320). The heterodimer displaying a signal with HIV1_1 target (SEQ ID NO:319) is observed at positions D3. On the filter, the position of mutants in certain positions as an example is: line C, SEQ ID NO:10; line D, SEQ ID NO:11; column 2, SEQ ID NO:1; column 3, SEQ ID NO:2; column 4; SEQ ID NO:5. These mutants have been described in Tables II and IV. Each cluster contains 6 spots. On the 4 spots on the left, a yeast strain harboring the HIV1_1 target (SEQ ID NO:319) has been mated with another yeast strain containing the meganuclease variants. The two spots on the right contain the same negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3)

Figure 14: Cleavage of HIV1_1.3 (SEQ ID NO:321) and HIV1_1.5 (SEQ ID NO:323) targets by meganuclease variants improved by random mutagenesis in example 5. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_1.3 and HIV1_1.5 targets. On the filter, the positive variants presented correspond to: F3, SEQ ID NO:27; C11, SEQ ID NO:26; H8, SEQ ID NO:28; E12, SEQ ID NO:29; all described in Table VIII. Each cluster contains 6 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_1.3 (SEQ ID NO:321) or the HIV1_1.5 (SEQ ID NO:323) targets have been mated with another yeast strain containing the meganuclease variants. The two spots in the middle contain, as an internal control, a non-improved variant cleaving the HIV1_1.3 target (SEQ ID NO:321). The two spots on the right contain the same negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3).

Figure 15: Cleavage of HIV1_1 target (SEQ ID NO:319) by meganuclease variants improved by random mutagenesis in example 5. The figure displays an example of screening of I-*CreI* meganuclease variants with the HIV1_1 target, when mated with a meganuclease (SEQ ID NO:46) cleaving the HIV1_1.4 target. On the filter, the positive variants presented correspond to: F3, SEQ ID NO:27; C11, SEQ ID NO:26; H8, SEQ ID NO:28; E12, SEQ ID NO:29; all described in Table VIII. Each cluster contains 6 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_1.4 mutant and the HIV1_1 target have been mated with another yeast strain containing the meganuclease variants. The two spots in the middle contain, as an internal control, a non-improved variant. The two spots on the right contain the same negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3).

Figure 16: Cleavage of HIV1_1.3 (SEQ ID NO:321) and HIV1_1.5 (SEQ ID NO:323) targets by meganuclease variants improved by a second round of random mutagenesis in example 5bis. The figure displays an example of screening of I-*CreI* meganuclease variants with the HIV1_1.3 and HIV1_1.5 targets. On the filter, the positive variants presented correspond to: A12, SEQ ID NO:42; D8, SEQ ID NO:38; G8, SEQ ID NO:36; G3, SEQ ID NO:40; all described in Table IX. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_1.3 or the HIV1_1.5 targets have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, a variant issued from the first round of improvement.

Figure 17: Cleavage of HIV1_1 (SEQ ID NO:319) target by meganuclease variants improved by a second round of random mutagenesis in example 5bis. The figure displays an example of screening of I-*CreI* meganuclease variants with the HIV1_1 target, when mated with a meganuclease (SEQ ID NO:46) cleaving the HIV1_1.4 target. On the filter, the positive variants presented correspond to: A12, SEQ ID NO:42; D8, SEQ ID NO:38; G8, SEQ ID NO:36; G3, SEQ ID

NO:40; all described in Table IX. Each cluster contains 6 spots. On the 4 spots on the left, a yeast strain harboring the HIV1_1.4 mutant (SEQ ID NO:46) and the HIV1_1 target (SEQ ID NO:319) have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, a variant issued from the first round of improvement.

Figure 18: Cleavage of HIV1_1.3 (SEQ ID NO:321) and HIV1_1.5 (SEQ ID NO:323) targets by meganuclease variants improved by site-directed mutagenesis in example 6. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_1.3 and HIV1_1.5 targets. On the filter, the positive variants presented correspond to: F10, SEQ ID NO:63; H2, SEQ ID NO:60; H3, SEQ ID NO:59; A3, SEQ ID NO:64; F4, SEQ ID NO:65; some of them described in Table XI. Some of these variants show no cleavage activity as homodimers while they are active as heterodimers on the HIV1_1 target (see Figure 19). This is due to the presence of the G19S mutation in these variants. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_1.3 or the HIV1_1.5 targets have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, a variant issued from the first round of improvement.

Figure 19: Cleavage of HIV1_1 target (SEQ ID NO:319) by meganuclease variants improved by site-directed mutagenesis in example 6. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_1 target, when mated with a meganuclease (SEQ ID NO:46) cleaving the HIV1_1.4 target. On the filter, the positive variants presented correspond to: F10, SEQ ID NO:63; H2, SEQ ID NO:60; H3, SEQ ID NO:59; A3, SEQ ID NO:64; F4, SEQ ID NO:65; some of them described in Table XI. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_1.4 mutant and the

HIV1_1 target have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, a variant issued from the first round of improvement.

Figure 20: Cleavage of HIV1_1.4 (SEQ ID NO:322) and HIV1_1.6 (SEQ ID NO:324) targets by meganuclease variants improved by random mutagenesis in example 7. The figure displays an example of screening of I-*CreI* meganuclease variants with the HIV1_1.4 and HIV1_1.6 targets. On the filter, the positive variants presented correspond to: B7, SEQ ID NO:46; B9, SEQ ID NO:68; B12, SEQ ID NO:69; A9, SEQ ID NO:70; E5, SEQ ID NO:71; all described in Table XIII. Each cluster contains 6 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_1.4 or the HIV1_1.6 targets have been mated with another yeast strain containing the meganuclease variants. The two spots in the middle contain, as an internal control, a non-improved variant cleaving the HIV1_1.4 target. The two spots on the right contain the same negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3).

Figure 21: Cleavage of HIV1_1 target (SEQ ID NO:319) by meganuclease variants improved by random mutagenesis in example 7. The figure displays an example of screening of I-*CreI* meganuclease variants with the HIV1_1 target, when mated with a meganuclease (SEQ ID NO:26) cleaving the HIV1_1.3 target. On the filter, the positive variants presented correspond to: B7, SEQ ID NO:46; B9, SEQ ID NO:68; B12, SEQ ID NO:69; A9, SEQ ID NO:70; E5, SEQ ID NO:71; all described in Table XIII. Each cluster contains 6 spots. On the 2 spots on the left, as well as those in the middle, a yeast strain harboring the HIV1_1.3 mutant (SEQ ID NO:26) and the HIV1_1 target have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control

(i.e. cluster A3). The spot in the upper-right contains, as an internal control, a variant issued from the first round of improvement.

Figure 22: Cleavage of HIV1_1.4 (SEQ ID NO:322) and HIV1_1.6 (SEQ ID NO:324) targets by meganuclease variants improved by a second round of random mutagenesis in example 7bis. The figure displays an example of screening of I-*CreI* meganuclease variants with the HIV1_1.4 and HIV1_1.6 targets. On the filter, the positive variants presented correspond to: A3, SEQ ID NO:76; B1, SEQ ID NO:77; C1, SEQ ID NO:78; D3, SEQ ID NO:79; D5, SEQ ID NO:80; all described in Table XIV. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_1.4 or the HIV1_1.6 targets have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, a variant issued from the first round of improvement.

Figure 23: Cleavage of HIV1_1 target (SEQ ID NO:319) by meganuclease variants improved by a second round of random mutagenesis in example 7bis. The figure displays an example of screening of I-*CreI* meganuclease variants with the HIV1_1 target, when mated with a meganuclease (SEQ ID NO:26) cleaving the HIV1_1.3 target. On the filter, the positive variants presented correspond to: A3, SEQ ID NO:76; B1, SEQ ID NO:77; C1, SEQ ID NO:78; D3, SEQ ID NO:79; D5, SEQ ID NO:80; all described in Table XIV. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_1.3 mutant (SEQ ID NO:26) and the HIV1_1 target have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, a variant issued from the first round of improvement.

Figure 24: The HIV1_3 target sequence (SEQ ID NO:325) and its derivatives. In the HIV1_3.2 target (SEQ ID NO:326), the TTTA sequence in the middle of the target is replaced with GTAC, the bases found in C1221 (SEQ ID

NO:343). HIV1_3.3 (SEQ ID NO:327) is the palindromic sequence derived from the left part of HIV1_3.2, and HIV1_3.4 (SEQ ID NO:328) is the palindromic sequence derived from the right part of HIV1_3.2. HIV1_3.5 (SEQ ID NO:329) and HIV1_3.6 (SEQ ID NO:330) are pseudo-palindromic targets derived, respectively, from
 5 HIV1_3.3 and HIV1_3.4, containing the natural TTTA sequence in the middle of the target. As shown in the Figure, the boxed motives from 10CAG_P, 10ACA_P, 5CCT_P and 5GAC_P are found in the HIV1_3 series of targets.

Figure 25: Cleavage of HIV1_3.3 target (SEQ ID NO:327) by combinatorial variants. The figure displays an example of screening of I-CreI combinatorial variants with the HIV1_3.3 target. On the filter, the positive variants correspond to: A6, SEQ ID NO:89; A1, SEQ ID NO:91; A8, SEQ ID NO:90; A4, SEQ ID NO:88; all described in Table XVI. Each cluster contains 4 spots. On the spots on the left, a yeast strain harboring the HIV1_3.3 target has been mated with another yeast strain containing the meganuclease variants. The two spots on the right
 15 contain the same negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3).

Figure 26: Cleavage of HIV1_3.4 (SEQ ID NO:328) and HIV1_3.6 (SEQ ID NO:330) targets by combinatorial variants. The figure displays an example of screening of I-CreI combinatorial variants with the HIV1_3.4 and HIV1_3.6
 20 targets. On the filter, the positive variants correspond to: C12, SEQ ID NO:98; C8, SEQ ID NO:99; E4, SEQ ID NO:100; G4, SEQ ID NO:97; E9, SEQ ID NO:101; all described in Table XVIII. Each cluster contains 4 spots. On the spots on the left, a yeast strain harboring the HIV1_3.4 or the HIV1_3.6 targets has been mated with another yeast strain containing the meganuclease variants. The two spots on the right
 25 contain the same negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3).

Figure 27: Cleavage of HIV1_3.3 target (SEQ ID NO:327) by meganuclease variants improved by random mutagenesis in example 12. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_3.3 target. On the filter, the positive variants presented correspond to: E1, SEQ ID

NO:105; C8, SEQ ID NO:106; A2, SEQ ID NO:107; A7, SEQ ID NO:108; B10, SEQ ID NO:109; all described in Table XIX. Each cluster contains 6 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_3.3 target has been mated with another yeast strain containing the meganuclease variants. The two spots in the middle contain, as an internal control, a non-improved variant cleaving the HIV1_3.3 target. The two spots on the right contain the same negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3).

Figure 28: Cleavage of HIV1_3.3 target (SEQ ID NO:327) by meganuclease variants improved by a second round of random mutagenesis in example 12bis. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_3.3 target. On the filter, the positive variants presented correspond to: A11, SEQ ID NO:115; B7, SEQ ID NO:116; F12, SEQ ID NO:117; G2, SEQ ID NO:118; H9, SEQ ID NO:119; all described in Table XX. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_3.3 target has been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, a variant issued from the first round of improvement.

Figure 29: Cleavage of HIV1_3.3 (SEQ ID NO:327) and HIV1_3.5 (SEQ ID NO:329) targets by meganuclease variants improved by site-directed mutagenesis in example 13. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_3.3 and HIV1_3.5 targets. On the filter, the positive variants presented correspond to: A1, SEQ ID NO:126; G3, SEQ ID NO:127; C1, SEQ ID NO:128; H6, SEQ ID NO:129; E5, SEQ ID NO:130; described in Table XXI. Some of these variants show no cleavage activity as homodimers while they are active as heterodimers on the HIV1_3 target (see Figure 30). This is due to the presence of the G19S mutation in these variants. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_3.3 or the HIV1_3.5 targets have

been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, a previously improved variant.

Figure 30: Cleavage of HIV1_3 (SEQ ID NO:325) target by meganuclease variants improved by site-directed mutagenesis in example 13. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_3 target, when mated with a meganuclease (SEQ ID NO:125) cleaving the HIV1_3.4 target. On the filter, the positive variants presented correspond to: A1, SEQ ID NO:126; G3, SEQ ID NO:127; C1, SEQ ID NO:128; H6, SEQ ID NO:129; E5, SEQ ID NO:130; described in Table XXI. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_3.4 mutant (SEQ ID NO:125) and the HIV1_3 target have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, a previously improved variant.

Figure 31: Cleavage of HIV1_3.4 (SEQ ID NO:328) and HIV1_3.6 (SEQ ID NO:330) targets by meganuclease variants improved by random mutagenesis in example 14. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_3.4 and HIV1_3.6 targets. On the filter, the positive variants presented correspond to: E8, SEQ ID NO:136; B12, SEQ ID NO:137; B1, SEQ ID NO:138; B8, SEQ ID NO:139; D6, SEQ ID NO:140; all described in Table XXII. Each cluster contains 6 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_3.4 or the HIV1_3.6 targets has been mated with another yeast strain containing the meganuclease variants. The two spots in the middle contain, as an internal control, a non-improved variant cleaving the HIV1_3.4 target. The two spots on the right contain the same negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3).

Figure 32: Cleavage of HIV1_3.4 (SEQ ID NO:328) and HIV1_3.6 (SEQ ID NO:330) targets by meganuclease variants improved by a second round of random mutagenesis in example 14bis. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_3.4 and HIV1_3.6 targets. On the filter, the positive variants presented correspond to: F7, SEQ ID NO:146; B12, SEQ ID NO:147; G7, SEQ ID NO:148; D2, SEQ ID NO:149; A5, SEQ ID NO:150; all described in Table XXIII. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_3.4 or the HIV1_3.6 targets have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, a previously improved variant.

Figure 33: Cleavage of HIV1_3.4 (SEQ ID NO:328) and HIV1_3.6 (SEQ ID NO:330) targets by meganuclease variants improved by site-directed mutagenesis in example 15. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_3.4 and HIV1_3.6 targets. On the filter, the positive variants presented correspond to: D1, SEQ ID NO:156; C2, SEQ ID NO:157; F2, SEQ ID NO:158; A4, SEQ ID NO:159; G7, SEQ ID NO:160; described in Table XXIV. Each cluster contains 6 spots. On the 4 spots on the left, a yeast strain harboring the HIV1_3.4 or the HIV1_3.6 targets have been mated with another yeast strain containing 4 different meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, a non-improved variant.

Figure 34: Cleavage of HIV1_3 target (SEQ ID NO:325) by meganuclease variants improved by site-directed mutagenesis in example 15. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_3 target, when mated with a meganuclease (SEQ ID NO:109) cleaving the HIV1_3.3 target. On the filter, the positive variants presented correspond to: D1, SEQ ID NO:156; C2, SEQ ID NO:157; F2, SEQ ID NO:158; A4, SEQ ID NO:159; G7,

SEQ ID NO:160; described in Table XXIV. Each cluster contains 6 spots. On the 4 spots on the left, a yeast strain harboring the HIV1_3 target and the HIV1_3.3 mutant has been mated with another yeast strain containing different meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are
 5 serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, a previously improved variant.

Figure 35: The HIV1_4 (SEQ ID NO:331) target sequence and its derivatives. In the HIV1_4.2 target (SEQ ID NO:332), the GGAC sequence in the
 10 middle of the target is replaced with GTAC, the bases found in C1221 (SEQ ID NO:343). HIV1_4.3 (SEQ ID NO:333) is the palindromic sequence derived from the left part of HIV1_4.2, and HIV1_4.4 (SEQ ID NO:334) is the palindromic sequence derived from the right part of HIV1_4.2. HIV1_4.5 (SEQ ID NO:335) and HIV1_4.6 (SEQ ID NO:336) are pseudo-palindromic targets derived, respectively, from
 15 HIV1_4.3 and HIV1_4.4, containing the natural GGAC sequence in the middle of the target. As shown in the Figure, the boxed motives from 10AGC_P, 10TGT_P, 5TCT_P and 5TAT_P are found in the HIV1_4 series of targets.

Figure 36: Cleavage of HIV1_4.3 (SEQ ID NO:333) target by combinatorial variants. The figure displays an example of screening of I-CreI combi-
 20 natorial variants with the HIV1_4.3 target. On the filter, the positive variants correspond to: A11, SEQ ID NO:168; A5, SEQ ID NO:170; A2, SEQ ID NO:171; A4, SEQ ID NO:173; A3, SEQ ID NO:174; all described in Table XXVI. Each cluster contains 4 spots. On the spots on the left, a yeast strain harboring the HIV1_4.3 target has been mated with another yeast strain containing the meganuclease variants. The
 25 two spots on the right contain the same negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3).

Figure 37: Cleavage of HIV1_4.4 (SEQ ID NO:334) and HIV1_4.6 (SEQ ID NO:336) targets by combinatorial variants. The figure displays an example
 30 of screening of I-CreI combinatorial variants with the HIV1_4.4 and HIV1_4.6 targets. On the filter, the positive variants correspond to: A7, SEQ ID NO:177; A5, SEQ ID NO:178; B8, SEQ ID NO:179; E6, SEQ ID NO:180; F2, SEQ ID NO:181; all

described in Table XXVIII. Each cluster contains 4 spots. On the spots on the left, a yeast strain harboring the HIV1_4.4 or the HIV1_4.6 targets has been mated with another yeast strain containing the meganuclease variants. The two spots on the right contain the same negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3).

Figure 38: Cleavage of the HIV1_4.2 (SEQ ID NO:332) and HIV1_4 (SEQ ID NO:331) target sequences by heterodimeric combinatorial variants. Example of screening of combinations of I-CreI variants against the HIV1_4.2 target. Some heterodimers resulted in cleavage of the HIV1_4.2 target, while no cleavage activity was detected on the HIV1_4 target. On the filter, the position of mutants in certain positions as an example is: line A, SEQ ID NO:170; line B, SEQ ID NO:171; column 1, SEQ ID NO:177; column 2, SEQ ID NO:178; column 3; SEQ ID NO:179. These mutants have been described in Tables XXVI and XXVIII. Each cluster contains 6 spots. On the 4 spots on the left, a yeast strain harboring the HIV1_4 or HIV1_4.2 target have been mated with another yeast strain containing the meganuclease variants. The two spots on the right contain the same negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3).

Figure 39: Cleavage of HIV1_4.3 (SEQ ID NO:333) and HIV1_4.5 (SEQ ID NO:335) targets by meganuclease variants improved by random mutagenesis in example 20. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_4.3 and HIV1_4.5 targets. On the filter, the positive variants presented correspond to: F8, SEQ ID NO:189; C6, SEQ ID NO:190; E12, SEQ ID NO:191; G12, SEQ ID NO:192; G6, SEQ ID NO:193; G11, SEQ ID NO:194; all described in Table XXX. Each cluster contains 6 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_4.3 or the HIV1_4.5 targets have been mated with another yeast strain containing the meganuclease variants. The two spots in the middle contain, as an internal control, a non-improved variant cleaving the HIV1_4.3 target. The two spots on the right contain the same negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e.

cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3).

Figure 40: Cleavage of HIV1_4.3 (SEQ ID NO:333) and HIV1_4.5 (SEQ ID NO:335) targets by meganuclease variants improved by a second round of random mutagenesis in example 20bis. The figure displays an example of screening of I-*CreI* meganuclease variants with the HIV1_4.3 and HIV1_4.5 targets. On the filter, the positive variants presented correspond to: E7, SEQ ID NO:200; A1, SEQ ID NO:201; E9, SEQ ID NO:202; A4, SEQ ID NO:203; A11, SEQ ID NO:204; all described in Table XXXI. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_4.3 or the HIV1_4.5 targets has been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, an improved variant.

Figure 41: Cleavage of HIV1_4 (SEQ ID NO:331) target by meganuclease variants improved by a second round of random mutagenesis in example 20bis. The figure displays an example of screening of I-*CreI* meganuclease variants with the HIV1_4 target, when mated with a meganuclease (SEQ ID NO:199) cleaving the HIV1_4.4 target. On the filter, the positive variants presented correspond to: E7, SEQ ID NO:200; A1, SEQ ID NO:201; E9, SEQ ID NO:202; A4, SEQ ID NO:203; A11, SEQ ID NO:204; all described in Table XXXI. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_4.4 mutant (SEQ ID NO:199) and the HIV1_4 target have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, an improved variant.

Figure 42: Cleavage of HIV1_4 (SEQ ID NO:331) target by meganuclease variants improved by site-directed mutagenesis in example 21. The figure displays an example of screening of I-*CreI* meganuclease variants with the

HIV1_4 target, when mated with a meganuclease (SEQ ID NO:210) cleaving the HIV1_4.4 target. On the filter, the positive variants presented correspond to: A1, SEQ ID NO:211; A2, SEQ ID NO:212; A5, SEQ ID NO:213; A7, SEQ ID NO:214; A8, SEQ ID NO:215; G2, SEQ ID NO:216; described in Table XXXII. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_4.4 mutant (SEQ ID NO:210) and the HIV1_4 target have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, an improved variant.

Figure 43: Cleavage of HIV1_4.3 (SEQ ID NO:333) and HIV1_4.5 (SEQ ID NO:335) targets by meganuclease variants improved by site-directed mutagenesis in example 21. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_4.3 and HIV1_4.5 targets. On the filter, the variants presented correspond to: A1, SEQ ID NO:211; A2, SEQ ID NO:212; A5, SEQ ID NO:213; A7, SEQ ID NO:214; A8, SEQ ID NO:215; G2, SEQ ID NO:216; described in Table XXXII. Some of these variants show no cleavage activity as homodimers while they are active as heterodimers on the HIV1_4 target (see Figure 42). This is due to the presence of the G19S mutation in these variants. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_4.3 or the HIV1_4.5 targets have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, an improved variant.

Figure 44: Cleavage of HIV1_4.4 (SEQ ID NO:334) and HIV1_4.6 (SEQ ID NO:336) targets by meganuclease variants improved by random mutagenesis in example 22. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_4.4 and HIV1_4.6 targets. On the filter, the positive variants presented correspond to: D4, SEQ ID NO:199; D5, SEQ ID NO:210; C8, SEQ ID

NO:221; C10, SEQ ID NO:222; E8, SEQ ID NO:223; all described in Table XXXIII. Each cluster contains 6 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_4.4 or the HIV1_4.6 targets have been mated with another yeast strain containing the meganuclease variants. The two spots in the middle contain, as an
 5 internal control, a non-improved variant cleaving the HIV1_4.4 target. The two spots on the right contain the same negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3).

Figure 45: Cleavage of HIV1_4 (SEQ ID NO:331) target by
 10 meganuclease variants improved by random mutagenesis in example 22. The figure displays an example of screening of I-*CreI* meganuclease variants with the HIV1_4 target, when mated with a meganuclease (SEQ ID NO:190) cleaving the HIV1_4.3 target. On the filter, the positive variants presented correspond to: D4, SEQ ID NO:199; D5, SEQ ID NO:210; C8, SEQ ID NO:221; C10, SEQ ID NO:222; E8, SEQ
 15 ID NO:223; all described in Table XXXIII. Each cluster contains 6 spots. On the 4 spots on the left, a yeast strain harboring the HIV1_4.3 mutant (SEQ ID NO:190) and the HIV1_4 target have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative
 20 control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, a non-improved variant.

Figure 46: Cleavage of HIV1_4 (SEQ ID NO:331) target by
 meganuclease variants improved by site-directed mutagenesis in example 23. The
 25 figure displays an example of screening of I-*CreI* meganuclease variants with the HIV1_4 target, when mated with a meganuclease (SEQ ID NO:190) cleaving the HIV1_4.3 target. On the filter, the positive variants presented correspond to: B5, SEQ ID NO:229; B4, SEQ ID NO:231; A5, SEQ ID NO:235; A8, SEQ ID NO:236; A11, SEQ ID NO:237; described in Table XXXIV. Each cluster contains 4 spots. On the 2
 30 spots on the left, a yeast strain harboring the HIV1_4 target and the HIV1_4.3 mutant (SEQ ID NO:190) has been mated with another yeast strain containing different meganuclease variants. The spot on the low-right contain negative or positive

controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, an improved variant.

5 Figure 47: Cleavage of HIV1_4.4 (SEQ ID NO:334) and HIV1_4.6 (SEQ ID NO:336) targets by meganuclease variants improved by site-directed mutagenesis in example 23. The figure displays an example of screening of I-*CreI* meganuclease variants with the HIV1_4.4 and HIV1_4.6 targets. On the filter, the positive variants presented correspond to: B5, SEQ ID NO:229; B4, SEQ ID NO:231; 10 A5, SEQ ID NO:235; A8, SEQ ID NO:236; A11, SEQ ID NO:237; described in Table XXXIV. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_4.4 or the HIV1_4.6 targets have been mated with another yeast strain containing different meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters 15 as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, an improved variant.

 Figure 48: The HIV1_5 target sequence (SEQ ID NO:337) and its derivatives. In the HIV1_5.2 target (SEQ ID NO:338), the ATAC sequence in the 20 middle of the target is replaced with GTAC, the bases found in C1221 (SEQ ID NO:343). HIV1_5.3 (SEQ ID NO:339) is the palindromic sequence derived from the left part of HIV1_5.2, and HIV1_5.4 (SEQ ID NO:340) is the palindromic sequence derived from the right part of HIV1_5.2. HIV1_5.5 (SEQ ID NO:341) and HIV1_5.6 (SEQ ID NO:342) are pseudo-palindromic targets derived, respectively, from 25 HIV1_5.3 and HIV1_5.4, containing the natural ATAC sequence in the middle of the target. As shown in the Figure, the boxed motives from 10TCT_P, 10CTG_P, 5TAG_P and 5CCT_P are found in the HIV1_5 series of targets.

 Figure 49: Cleavage of HIV1_5.3 (SEQ ID NO:339) target by combinatorial variants. The figure displays an example of screening of I-*CreI* combi- 30 natorial variants with the HIV1_5.3 target. On the filter, the two positive variants correspond to: A1, SEQ ID NO:242; A2, SEQ ID NO:241; described in Table XXXVI. Each cluster contains 4 spots. On the spots on the left, a yeast strain

harboring the HIV1_5.3 target has been mated with another yeast strain containing the meganuclease variants. The two spots on the right contain the same negative or positive controls. These controls are: negative control (cluster A1), positive control (cluster A2), and strong positive control (cluster A3).

5 Figure 50: Cleavage of HIV1_5.4 (SEQ ID NO:340) target by combinatorial variants. The figure displays an example of screening of I-*CreI* combinatorial variants with the HIV1_5.4 target. On the filter, the positive variants correspond to: A1, SEQ ID NO:249; A3, SEQ ID NO:245; A4, SEQ ID NO:252; A7, SEQ ID NO:250; A10, SEQ ID NO:246; all described in Table XXXVIII. Each
10 cluster contains 4 spots. On the spots on the left, a yeast strain harboring the HIV1_5.4 target has been mated with another yeast strain containing the meganuclease variants. The two spots on the right contain the same negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster
15 A3).

 Figure 51: Cleavage of the HIV1_5.2 target sequence (SEQ ID NO:338) by heterodimeric combinatorial variants. Example of screening of combinations of I-*CreI* variants against the HIV1_5.2 target. One heterodimer resulted in cleavage of the HIV1_5.2 target. The heterodimer displaying a signal with HIV1_5.2
20 target is observed at position B4. On the filter, the position of certain mutants as an example is: line A, SEQ ID NO:242; line B, SEQ ID NO:241; column 3, SEQ ID NO:245; column 4, SEQ ID NO:252; column 5, SEQ ID NO:251. These mutants have been described in Tables XXXVI and XXXVIII. Each cluster contains 6 spots. On the
25 4 spots on the left, a yeast strain harboring the HIV1_5.2 target has been mated with another yeast strain containing the meganuclease variants. The two spots on the right contain the same negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3).

 Figure 52: Cleavage of HIV1_5.3 target (SEQ ID NO:339) by
30 meganuclease variants improved by random mutagenesis in example28. The figure displays an example of screening of I-*CreI* meganuclease variants with the HIV1_5.3 target. On the filter, the positive variants presented correspond to: A6, SEQ ID

NO:256; A12, SEQ ID NO:257; A11, SEQ ID NO:258; A10, SEQ ID NO:259; A2, SEQ ID NO:260 ;all described in Table XXXIX. Each cluster contains 6 spots. On the 4 spots on the left, a yeast strain harboring the HIV1_5.3 or target has been mated with another yeast strain containing the meganuclease variants. The spot on the low-
 5 right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, a non-improved variant.

Figure 53: Cleavage of HIV1_5.3 (SEQ ID NO:339) and HIV1_5.5
 10 (SEQ ID NO:341) targets by meganuclease variants improved by a second round of random mutagenesis in example 28bis. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_5.3 and HIV1_5.5 targets. On the filter, the positive variants presented correspond to: G2, SEQ ID NO:266; E4, SEQ ID NO:267; C2, SEQ ID NO:268; A12, SEQ ID NO:269; C11, SEQ ID NO:270; all
 15 described in Table XL. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_5.3 or the HIV1_5.5 targets have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2),
 20 and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, an improved variant.

Figure 54: Cleavage of HIV1_5 target (SEQ ID NO:337) by meganuclease variants improved by a second round of random mutagenesis in example 28bis. The figure displays an example of screening of I-CreI meganuclease
 25 variants with the HIV1_5 target, when mated with a meganuclease (SEQ ID NO:276) cleaving the HIV1_5.4 target. On the filter, the positive variants presented correspond to: G2, SEQ ID NO:266; E4, SEQ ID NO:267; C2, SEQ ID NO:268; A12, SEQ ID NO:269; C11, SEQ ID NO:270; all described in Table XL. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_5.4 mutant and the
 30 HIV1_5 target have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e.

cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, an improved variant.

Figure 55: Cleavage of HIV1_5.3 (SEQ ID NO:339) and HIV1_5.5 (SEQ ID NO:341) targets by meganuclease variants improved by site-directed mutagenesis in example 29. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_5.3 and HIV1_5.5 targets. On the filter, the positive variants presented correspond to: C6, SEQ ID NO:278; F8, SEQ ID NO:279; H7, SEQ ID NO:280; F1, SEQ ID NO:281; G12, SEQ ID NO:282; described in Table XLI. Some of these variants show no cleavage activity as homodimers while they are active as heterodimers on the HIV1_5 target (see Figure 56). This is due to the presence of the G19S mutation in these variants. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_5.3 or the HIV1_5.5 targets has been mated with another yeast strain containing the meganuclease variants. The spot on the low-right is a negative control. The spot in the upper-right contains, as an internal control, an improved variant.

Figure 56: Cleavage of HIV1_5 target (SEQ ID NO:337) by meganuclease variants improved by site-directed mutagenesis in example 29. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_5 target, when mated with a meganuclease (SEQ ID NO:276) cleaving the HIV1_5.4 target. On the filter, the positive variants presented correspond to: C6, SEQ ID NO:278; F8, SEQ ID NO:279; H7, SEQ ID NO:280; F1, SEQ ID NO:281; G12, SEQ ID NO:282; described in Table XLI. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_5.4 mutant (SEQ ID NO:276) and the HIV1_5 target has been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, an improved variant.

Figure 57: Cleavage of HIV1_5.4 (SEQ ID NO:340) and HIV1_5.6 (SEQ ID NO:342) targets by meganuclease variants improved by random mutagenesis in example 30. The figure displays an example of screening of I-CreI meganuclease

variants with the HIV1_5.4 and HIV1_5.6 targets. On the filter, the positive variants presented correspond to: D6, SEQ ID NO:276; A4, SEQ ID NO:288; C10, SEQ ID NO:289; A9, SEQ ID NO:290; A1, SEQ ID NO:291; all described in Table XLII. Each cluster contains 6 spots. On the 4 spots on the left, a yeast strain harboring the HIV1_5.4 or the HIV1_5.6 targets has been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, a non-improved variant cleaving the HIV1_5.4 target.

Figure 58: Cleavage of HIV1_5.4 (SEQ ID NO:340) and HIV1_5.6 (SEQ ID NO:342) targets by meganuclease variants improved by a second round of random mutagenesis in example 30bis. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_5.4 and HIV1_5.6 targets. On the filter, the positive variants presented correspond to: A12, SEQ ID NO:297; A1, SEQ ID NO:298; A11, SEQ ID NO:299; A8, SEQ ID NO:300; B4, SEQ ID NO:301; all described in Table XLIII. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_5.4 or the HIV1_5.6 targets have been mated with another yeast strain containing the meganuclease variants. The spot on the low-right contain negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, an improved variant.

Figure 59: Cleavage of HIV1_5.4 (SEQ ID NO:340) and HIV1_5.6 (SEQ ID NO:342) targets by meganuclease variants improved by site-directed mutagenesis in example 31. The figure displays an example of screening of I-CreI meganuclease variants with the HIV1_5.4 and HIV1_5.6 targets. On the filter, the positive variants presented correspond to: H1, SEQ ID NO:307; H2, SEQ ID NO:308; H9, SEQ ID NO:309; B3, SEQ ID NO:310; H3, SEQ ID NO:311; described in Table XLIV. Each cluster contains 4 spots. On the 2 spots on the left, a yeast strain harboring the HIV1_5.4 or the HIV1_5.6 targets has been mated with another yeast strain containing different meganuclease variants. The spot on the low-right contains

negative or positive controls. These controls are serially repeated every three clusters as follows: negative control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control (i.e. cluster A3). The spot in the upper-right contains, as an internal control, an improved variant.

5 Figure 60: Cleavage of HIV1_5 (SEQ ID NO:337) target by
meganuclease variants improved by site-directed mutagenesis in example 31. The
figure displays an example of screening of I-CreI meganuclease variants with the
HIV1_5 target, when mated with a meganuclease (SEQ ID NO:256) cleaving the
HIV1_5.3 target. On the filter, the positive variants presented correspond to: H1, SEQ
10 ID NO:307; H2, SEQ ID NO:308; H9, SEQ ID NO:309; B3, SEQ ID NO:310; H3,
SEQ ID NO:311; described in Table XLIV. Each cluster contains 4 spots. On the 2
spots on the left, a yeast strain harboring the HIV1_5 target and the HIV1_5.3 mutant
(SEQ ID NO:256) has been mated with another yeast strain containing different
meganuclease variants. The spot on the low-right contain negative or positive
15 controls. These controls are serially repeated every three clusters as follows: negative
control (i.e. cluster A1), positive control (i.e. cluster A2), and strong positive control
(i.e. cluster A3). The spot in the upper-right contains, as an internal control, an
improved variant.

20 There will now be described by way of example a specific mode
contemplated by the Inventors. In the following description numerous specific details
are set forth in order to provide a thorough understanding. It will be apparent however,
to one skilled in the art, that the present invention may be practiced without limitation
to these specific details. In other instances, well known methods and structures have
not been described so as not to unnecessarily obscure the description.

25 **Example 1: Strategy for engineering meganucleases cleaving the HIV1_1 target
from the HIV1 virus**

30 The HIV1_1 target is a 22 bp (non-palindromic) target located in U3
region of the proviral LTRs (Figures 2 and 7). Since the LTRs are duplicated
sequences flanking the viral ORFs in the integrated provirus, the HIV1_1 target is
present twice in the HIV_1 provirus. This target is precisely located at positions 84-
105 and 8159-9180 of the HIV-1 pNL4-3 vector (accession number AF324493,
Adachi et al., J. Virol., 1986, 59, 284-291), this infective molecular clone was

generated from the NY5 strain (Barre-Sinoussi et al., Science, 1983, 220, 868-871 and Benn et al., Science, 1985, 230, 949-951) a subtype B infectious molecular clone.

The HIV1_1 sequence is partly a patchwork of the 10AGA_P, 10TGG_P, 5TAC_P and 5_CTG_P targets (these designations describe the 3bp
5 starting at the indicated nucleotide of the I-CreI target, for instance 10AGA_P indicates that nucleotides -10, -9 and -8 are A(-10) G(-9) A(-8) (Figure 7)) which are cleaved by previously identified meganucleases. These meganucleases were obtained as described in International PCT Applications WO 2006/097784 and WO 2006/097853; Arnould et al., J. Mol. Biol., 2006, 355, 443-458; Smith et al., Nucleic
10 Acids Res., 2006.

The 10AGA_P, 10TGG_P, 5TAC_P and 5_CTG_P target sequences are 24 bp derivatives of C1221, a palindromic sequence cleaved by I-CreI (Arnould et al., precited). However, the structure of I-CreI bound to its DNA target suggests that the two external base pairs of these targets (positions -12 and 12) have no impact on
15 binding and cleavage (Chevalier et al., Nat. Struct. Biol., 2001, 8, 312-316; Chevalier and Stoddard, Nucleic Acids Res., 2001, 29, 3757-3774; Chevalier et al., J. Mol. Biol., 2003, 329, 253-269), and in this study, only positions -11 to 11 were considered. Consequently, the HIV1_1 series of targets were defined as 22 bp sequences instead of 24 bp. HIV1_1 differs from C1221 (SEQ ID NO: 343) in the 4 bp central region.
20 According to the structure of the I-CreI protein bound to its target, there is no contact between the 4 central base pairs (positions -2 to 2) and the I-CreI protein (Chevalier et al., Nat. Struct. Biol., 2001, 8, 312-316; Chevalier and Stoddard, Nucleic Acids Res., 2001, 29, 3757-3774; Chevalier et al., J. Mol. Biol., 2003, 329, 253-269). Thus, the bases at these positions should not impact the binding efficiency. However, they could
25 affect cleavage, which results from two nicks at the edge of this region. Thus, the ACAC sequence in -2 to 2 was first substituted with the GTAC sequence from C1221, resulting in target HIV1_1.2 (Figure 7). Then, two palindromic targets, HIV1_1.3 and HIV1_1.4, were derived from HIV1_1.2 (Figure 7). Since HIV1_1.3 and HIV1_1.4 are palindromic, they should be cleaved by homodimeric proteins. Two other pseudo-
30 palindromic targets were derived from these two containing the ACAC sequence in -2 to 2 (targets HIV1_1.5 and HIV1_1.6, Figure 7). Thus, proteins able to cleave HIV1_1.3 and HIV1_1.4 targets or, preferentially, the pseudo-palindromic targets as

homodimers were first designed (examples 2 and 3) and then co-expressed to obtain heterodimers cleaving HIV1_1 (example 4). Heterodimers cleaving the HIV1_1.2 and HIV1_1 targets could be identified. In order to improve cleavage activity for the HIV1_1 target, a series of variants cleaving HIV1_1.3 and HIV1_1.4 was chosen, and then refined. The chosen variants were subjected to random or site-directed mutagenesis, and used to form novel heterodimers that were screened against the HIV1_1 target (examples 5, 6, 7 and 8). Heterodimers could be identified with an improved cleavage activity for the HIV1_1 target.

Example 2: Identification of meganucleases cleaving HIV1_1.3

This example shows that I-CreI variants can cut the HIV1_1.3 DNA target sequence derived from the left part of the HIV1_1.2 target in a palindromic form (Figure 7).

HIV1_1.3 is similar to 10AGA_P at positions ± 1 , ± 2 , ± 6 , ± 8 , ± 9 , and ± 10 and to 5TAC_P at positions ± 1 , ± 2 , ± 3 , ± 4 , ± 5 and ± 6 . It was hypothesized that positions ± 7 and ± 11 would have little effect on the binding and cleavage activity.

Variants able to cleave the 10AGA_P target were obtained by mutagenesis of I-CreI N75 or D75, at positions 28, 30, 32, 33, 38, 40 and 70, as described previously in Smith et al. Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156. Variants able to cleave 5TAC_P were obtained by mutagenesis on I-CreI N75 at positions 24, 44, 68, 70, 75 and 77 as described in Arnould et al., J. Mol. Biol., 2006, 355, 443-458; Smith et al. Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2006/097784, WO 2006/097853, WO 2007/060495 and WO 2007/049156.

Both sets of proteins are mutated at position 70. However, the existence of two separable functional subdomains was hypothesized. This implies that this position has little impact on the specificity at bases 10 to 8 of the target. Mutations at positions 24 found in variants cleaving the 5TAC_P target will be lost during the combinatorial process. But it was hypothesized that this will have little impact on the capacity of the combined variants to cleave the HIV1_1.3 target.

Therefore, to check whether combined variants could cleave the HIV1_1.3 target, mutations at positions 44, 68, 70, 75 and 77 from proteins cleaving 5TAC_P were combined with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10AGA_P.

A) Material and Methods

a) Construction of target vector

The target was cloned as follows: an oligonucleotide corresponding to the HIV1_1.3 target sequence flanked by gateway cloning sequences was ordered from PROLIGO:

5 5' TGGCATACAAGTTTGCAGAACTACGTACGTAGTTCTGCCAATCGTCTGTCA 3'
(SEQ ID NO: 14). The same procedure was followed for cloning the HIV1_1.5 target, using the oligonucleotide:

5' TGGCATACAAGTTTGCAGAACTACACACGTAGTTCTGCCAATCGTCTGTCA 3'
(SEQ ID NO: 15). Double-stranded target DNA, generated by PCR amplification of
10 the single stranded oligonucleotide, was cloned using the Gateway protocol (INVITROGEN) into the yeast reporter vector (pCLS1055, Figure 8). Yeast reporter vector was transformed into *Saccharomyces cerevisiae* strain FYBL2-7B (*MAT a*, *ura3* Δ 851, *trp1* Δ 63, *leu2* Δ 1, *lys2* Δ 202), resulting in a reporter strain.

b) Construction of combinatorial mutants

15 I-*CreI* variants cleaving 10AGA_P or 5TAC_P were previously identified, as described in Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156, and Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; International PCT Applications WO 2006/097784 and WO 2006/097853, respectively for the 10AGA_P and 5TAC_P
20 targets. In order to generate I-*CreI* derived coding sequences containing mutations from both series, separate overlapping PCR reactions were carried out that amplify the 5' end (aa positions 1-43) or the 3' end (positions 39-167) of the I-*CreI* coding sequence. For both the 5' and 3' end, PCR amplification is carried out using primers (Gal10F 5'-gcaactttagtgctgacacatacagg-3' (SEQ ID NO: 16) or Gal10R 5'-
25 acaaccttgattggagactgacc-3'(SEQ ID NO: 17)) specific to the vector (pCLS0542, Figure 9) and primers (assF 5'-ctannnttgaccttt-3' (SEQ ID NO: 18) or assR 5'-aaagggtcaannntag-3'(SEQ ID NO: 19)), where nnn codes for residue 40, specific to the I-*CreI* coding sequence for amino acids 39-43.

30 The PCR fragments resulting from the amplification reaction using the same primers and with the same coding sequence for residue 40 were pooled. Then, each pool of PCR fragments resulting from the reaction with primers Gal10F and assR or assF and Gal10R was mixed in an equimolar ratio. Finally, approximately

25 ng of each final pool of the two overlapping PCR fragments and 75 ng of vector DNA (pCLS0542, Figure 9) linearized by digestion with *NcoI* and *EagI* were used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (MAT α , *trp1* Δ 63, *leu2* Δ 1, *his3* Δ 200) using a high efficiency LiAc transformation protocol (Gietz and
5 Woods, Methods Enzymol., 2002, 350, 87-96). An intact coding sequence containing both groups of mutations is generated by *in vivo* homologous recombination in yeast.

c) Mating of meganuclease expressing clones and screening in yeast

Screening was performed as described previously (Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458). Mating was performed using a colony gridder
10 (QpixII, GENETIX). Variants were gridded on nylon filters covering YPD plates, using a low gridding density (4-6 spots/cm²). A second gridding process was performed on the same filters to spot a second layer consisting of the reporter-harboring yeast strain. Membranes were placed on solid agar YPD rich medium, and incubated at 30 °C for one night, to allow mating. Next, filters were transferred to
15 synthetic medium, lacking leucine and tryptophan, with galactose (2 %) as a carbon source, and incubated for five days at 37 °C, to select for diploids carrying the expression and target vectors. After 5 days, filters were placed on solid agarose medium with 0.02 % X-Gal in 0.5 M sodium phosphate buffer, pH 7.0, 0.1 % SDS, 6% dimethyl formamide (DMF), 7 mM β -mercaptoethanol, 1% agarose, and
20 incubated at 37°C, to monitor β -galactosidase activity. Results were analyzed by scanning and quantification was performed using appropriate software.

d) Sequencing of variants

To recover the variant expression plasmids, yeast DNA was extracted using standard protocols and used to transform *E. coli*. Sequencing of
25 variant ORFs was then performed on the plasmids by MILLEGEN SA. Alternatively, ORFs were amplified from yeast DNA by PCR (Akada *et al.*, Biotechniques, 2000, 28, 668-670), and sequencing was performed directly on the PCR product by MILLEGEN SA.

B) Results

30 I-*CreI* combinatorial variants were constructed by associating mutations at positions 44, 68, 70, 75 and 77 from proteins cleaving 5TAC_P with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10AGA_P on the I-*CreI*

scaffold, resulting in a library of complexity 1600. Examples of combinatorial variants are displayed in Table I. In Table I the peptide sequence of these two subdomains are provided in the first column and second row respectively.

5 This library was transformed into yeast and 3348 clones (2 times the diversity) were screened for cleavage against the HIV1_1.3 and HIV1_1.5 DNA targets. 36 positive clones were found to cleave the HIV1_1.3 target, which after sequencing turned out to correspond to 31 different novel endonuclease variants (Table II). Those variants showed no cleavage activity of the HIV1_1.5 DNA target. Examples of positives are shown in Figure 10. Some of the variants obtained display
10 non parental combinations at positions 28, 30, 32, 33, 38, 40 or 44, 68, 70, 75, 77. Such combinations likely result from PCR artifacts during the combinatorial process. Alternatively, the variants may be I-CreI combined variants resulting from micro-recombination between two original variants during *in vivo* homologous recombination in yeast.

Table I: Panel of variants* theoretically present in the combinatorial library

Amino acids at positions 44, 68, 70, 75 and 77 (ex : ARNNI stands for A44, R68, N70, N75 and I77)	Amino acids at positions 28, 30,32, 33, 38 and 40 (ex: KHSSQS stands for K28, H30, S32, S33, Q38 and S40)										
	KTTYQS	KQSHQS	KNSCRS	KDSRQS	KGSYHN	KTSAQS	KTSHRS	KNSGGS	KNSPRS	KNSPKS	KSSGQS
ARSYT			+								
AYSHI											
YYSYR											
ARSRY											
AYSRV											
ARSRN											
NTSRY			+								
ARRNI											
NYSRV											
NTSRV											
NRSRI											
VERN R											
NKSRT											
NYSRY			+								
AYS RQ											
NTSRQ											
DNSNI											
NRSRN			+								
AYS RK											
NYSRI											
AASRI											
NRRNI											
ARSRV											
NTSRI											

*Only 264 out of the 1600 combinations are displayed.

+ indicates that a functional combinatorial variant cleaving the HIV1_1.3 target was found among the identified positives.

Table II: I-CreI variants with additional mutations capable of cleaving the HIV1_1.3 target

Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77 of the I-CreI variants (ex: KRSRES/TYSNI stands for K28, R30, S32, R33, E38, S40/ T44, Y68, S70, N75 and I77)	SEQ ID NO:
KGSYRS/NYSRI +93Q	1
KGSYRS/NYSRY	2
KGSYRS/VERNR +80K	3
KGSYRS/IERNR +80K	4
KGSYRS/NYSRQ	5
KNSCRS/AYSRQ +154N	6

Example 3: Making of meganucleases cleaving HIV1_1.4

- 5 This example shows that I-CreI variants can cleave the HIV1_1.4 DNA target sequence derived from the right part of the HIV1_1.2 target in a palindromic form (Figure 7).

HIV1_1.4 is similar to 5CTG_P at positions ± 1 , ± 2 , ± 3 , ± 4 , ± 5 and ± 8 and to 10TGG_P at positions ± 1 , ± 2 , ± 3 , ± 4 , ± 8 , ± 9 and ± 10 . It was hypothesized
 10 that positions ± 6 , ± 7 and ± 11 would have little effect on the binding and cleavage activity. Variants able to cleave 5CTG_P were obtained by mutagenesis of I-CreI N75 at positions 44, 68, 70, 75 and 77, as described previously (Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2006/097784, WO 2006/097853, WO
 15 2007/060495 and WO 2007/049156). Variants able to cleave the 10TGG_P target were obtained by mutagenesis of I-CreI N75 or D75, at positions 28, 30, 32, 33, 38, 40 and 70, as described previously in Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156.

Both sets of proteins are mutated at position 70. However, the
 20 existence of two separable functional subdomains was hypothesized. This implies that this position has little impact on the specificity at bases 10 to 8 of the target.

Therefore, to check whether combined variants could cleave the HIV1_1.4 target, mutations at positions 44, 68, 70, 75 and 77 from proteins cleaving

5TTC_P were combined with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10GGA_P.

A) Material and Methods

a) Construction of target vector

- 5 The experimental procedure is as described in example 2, with the exception that different oligonucleotides corresponding to the HIV1_1.4 and HIV1_1.6 targets. The oligonucleotide used for the HIV1_1.4 target was:

5'TGGCATACAAGTTTCCTGGCCCTGGTACCAGGGCCAGGCAATCGTCTGTCA 3'
(SEQ ID NO: 20),

- 10 and

5'TGGCATACAAGTTTCCTGGCCCTGACACCAGGGCCAGGCAATCGTCTGTCA 3'
(SEQ ID NO: 21) for HIV1_1.6 target.

b) Construction of combinatorial variants

- I-CreI variants cleaving 10TGG_P or 5CTG_P were previously
15 identified, as described in Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156, and Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; International PCT Applications WO 2006/097784 and WO 2006/097853, respectively for the 10TGG_P and 5CTG_P targets. In order to generate I-CreI derived coding sequences containing mutations
20 from both series, separate overlapping PCR reactions were carried out that amplify the 5' end (aa positions 1-43) or the 3' end (positions 39-167) of the I-CreI coding sequence. For both the 5' and 3' end, PCR amplification is carried out using primers (Gal10F 5'-gcaactttagtgctgacacatacagg-3' (SEQ ID NO: 16) or Gal10R 5'-acaaccttgattggagacttgacc-3' (SEQ ID NO: 17)) specific to the vector (pCLS1107, Figure 11) and primers (assF 5'-ctannnttgacctt-3' (SEQ ID NO: 18) or assR 5'-aaaggtcaannntag-3' (SEQ ID NO: 19)), where nnn codes for residue 40, specific to the I-CreI coding sequence for amino acids 39-43. The PCR fragments resulting from the amplification reaction realized with the same primers and with the same coding sequence for residue 40 were pooled. Then, each pool of PCR fragments resulting
25 from the reaction with primers Gal10F and assR or assF and Gal10R was mixed in an equimolar ratio. Finally, approximately 25 ng of each final pool of the two overlapping PCR fragments and 75 ng of vector DNA (pCLS1107, Figure 11)
30

linearized by digestion with DraIII and NgoMIV were used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (MAT α , trp1 Δ 63, leu2 Δ 1, his3 Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). An intact coding sequence containing both groups of mutations is generated by *in vivo* homologous recombination in yeast.

c) Mating of meganuclease expressing clones and screening in yeast

Screening was performed as described previously (Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458). Mating was performed using a colony gridder (QpixII, GENETIX). Variants were gridded on nylon filters covering YPD plates, using a low gridding density (4-6 spots/cm²). A second gridding process was performed on the same filters to spot a second layer consisting of the reporter-harboring yeast strain. Membranes were placed on solid agar YPD rich medium, and incubated at 30 °C for one night, to allow mating. Next, filters were transferred to synthetic medium, lacking tryptophan, adding G418, with galactose (2 %) as a carbon source, and incubated for five days at 37 °C, to select for diploids carrying the expression and target vectors. After 5 days, filters were placed on solid agarose medium with 0.02 % X-Gal in 0.5 M sodium phosphate buffer, pH 7.0, 0.1 % SDS, 6% dimethyl formamide (DMF), 7 mM β -mercaptoethanol, 1% agarose, and incubated at 37°C, to monitor β -galactosidase activity. Results were analyzed by scanning and quantification was performed using appropriate software. Positives resulting clones were verified by sequencing (MILLEGEN) as described in example 2.

B) Results

I-CreI combinatorial variants were constructed by associating mutations at positions 44, 68, 70, 75 and 77 from proteins cleaving 5CTG_P with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10TGG_P on the I-CreI scaffold, resulting in a library of complexity 1600. Examples of combinatorial variants are displayed in Table III. This library was transformed into yeast and 3348 clones (2 times the diversity) were screened for cleavage against the HIV1_1.4 and HIV1_1.6 DNA targets. A total of 32 positive clones were found to cleave HIV1_1.4. Sequencing of these 32 clones allowed the identification of 25 novel endonuclease variants. One of those variants showed cleavage activity on the HIV1_1.6 DNA target. Examples of positives are shown in Figure 12. The sequence of several of the variants

identified display non parental combinations at positions 28, 30, 32, 33, 38, 40 or 44, 68, 70, 75, 77 as well as additional mutations (see examples Table IV). Such variants likely result from PCR artifacts during the combinatorial process. Alternatively, the variants may be I-*CreI* combined variants resulting from micro-recombination

5 between two original variants during *in vivo* homologous recombination in yeast.

Table III: Panel of variants* theoretically present in the combinatorial library

Amino acids at positions 44, 68, 70, 75 and 77 (ex: ARNNI stands for A44, R68, N70, N75 and I77)	Amino acids at positions 28, 30, 32, 33, 38 and 40 (ex: KHSSQS stands for K28, H30, S32, S33, Q38 and S40)										
	ANSSRK	NNSSRR	QNSSRK	KNRGRS	KNSCAS	KYTCQS	KNSTGS	KNSTQT	KNSCHS	KSSSTS	KNTTQS
RYSDN					+						
RASER											
RQSER											
RYSEI											
RYSET					+						
KYSNI											
ATSNR											
RYSEY					+						
EYSES											
RTSER											
RYSTI											
RYSDR											
RYSDT					+				+		
SRSKE											
RRSEY											
RYSEV											
RYSER											
RYSDQ									+		
KYSEV											
KYSQT											
RYSNI											
RRSDY											
HYSNH											
PKSNL											

* Only 264 out of the 1600 combinations are displayed.

+ indicates that a functional combinatorial variant cleaving the HIV1_1.4 target was found among the identified positives.

Table IV: I-CreI variants with additional mutations capable of cleaving the HIV1_1.4 target.

Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77 of the I-CreI variants (ex: KRSRES/TYSNI stands for K28, R30, S32, R33, E38, S40/ T44, Y68, S70, N75 and I77)	SEQ ID NO:
QNSSRK/KYSES	7
KNSCAS/KYSES	8
KNSSRN/KYSES	9
KCSTQR/RYSdq	10
KNSTQK/RYSdN	11
KNSSQS/RSSDR	12

Example 4: Making of meganucleases cleaving HIV1_1.2 and HIV1_1

5 I-CreI variants able to cleave each of the palindromic HIV1_1.2 derived targets (HIV1_1.3 and HIV1_1.4) were identified in example 2 and example 3. Pairs of such variants (one cutting HIV1_1.3 and one cutting HIV1_1.4) were co-expressed in yeast. Upon co-expression, there should be three active molecular species, two homodimers, and one heterodimer. It was assayed whether the
10 heterodimers that should be formed, cut the HIV1_1.2 and the non palindromic HIV1_1 targets.

A) Materials and Methods**a) Construction of target vector**

The experimental procedure is as described in example 2, with the exception that an
15 oligonucleotide corresponding to the HIV1_1.2 target sequence:

5'TGGCATAACAAGTTTGCAGAACTACGTACCAGGGCCAGGCAATCGTCTGTCA 3'
(SEQ ID NO: 22) or the HIV1_1 target sequence:

5'TGGCATAACAAGTTTGCAGAACTACACACCAGGGCCAGGCAATCGTCTGTCA 3'
(SEQ ID NO: 23) was used.

20 b) Co-expression of variants

Yeast DNA was extracted from variants cleaving the HIV1_1.4 target in the pCLS1107 expression vector using standard protocols and was used to transform *E. coli*. The resulting plasmid DNA was then used to transform yeast strains expressing a variant cutting the HIV1_1.3 target in the pCLS0542 expression vector.

Transformants were selected on synthetic medium lacking leucine and containing G418.

c) Mating of meganucleases coexpressing clones and screening in yeast

Mating was performed using a colony gridder (QpixII, Genetix).

- 5 Variants were gridded on nylon filters covering YPD plates, using a low gridding density (4-6 spots/cm²). A second gridding process was performed on the same filters to spot a second layer consisting of different reporter-harboring yeast strains for each target. Membranes were placed on solid agar YPD rich medium, and incubated at 30°C for one night, to allow mating. Next, filters were transferred to synthetic
10 medium, lacking leucine and tryptophan, adding G418, with galactose (2 %) as a carbon source, and incubated for five days at 37 °C, to select for diploids carrying the expression and target vectors. After 5 days, filters were placed on solid agarose medium with 0.02 % X-Gal in 0.5 M sodium phosphate buffer, pH 7.0, 0.1 % SDS, 6% dimethyl formamide (DMF), 7mM β-mercaptoethanol, 1% agarose, and incubated
15 at 37°C, to monitor β-galactosidase activity. Results were analyzed by scanning and quantification was performed using appropriate software.

B) Results

- Co-expression of variants cleaving the HIV1_1.4 target (6 variants chosen among those described in Table III and Table IV) and six variants cleaving the
20 HIV1_1.3 target (described in Tables I and II) resulted in cleavage of the HIV1_1.2 target in most of the cases (Figure 13). Nevertheless, only one of these combinations was able to weakly cut the HIV1_1 natural target that differs from the HIV1_1.2 sequence by 2 bp at positions 1 and 2 (Figure 13). Examples of functional combinations are summarized in Table V and Table VI.

Table V: Cleavage of the HIV1_1.2 target by the heterodimeric variants

HIV1_1.2 target		Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77 of the I-CreI variants cleaving the HIV1_1.4 target (ex: KRSRES/TYSNI stands for K28, R30, S32, R33, E38, S40/ T44, Y68, S70, N75 and I77)	
		KNSTQK/RYS DN SEQ ID NO:11	KCSTQR/RYS DQ SEQ ID NO:10
Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77 Of I-CreI variants cleaving the HIV1_1.3 target (ex: KRGYQS/RHRDI stands for K28, R30, G32, Y33, Q38, S40/ R44, H68, R70, D75 and I77)	KGSYRS/NYSRQ SEQ ID NO:5	+	+
	KGSYRS/NYSRY +162P SEQ ID NO:13	+	+
	KGSYRS/NYSRY SEQ ID NO:2	+	+
	KGSYRS/NYSRI SEQ ID NO:1	+	+
	KGSYRS/VERNR SEQ ID NO:3	+	+
	KGSYRS/IERNR SEQ ID NO:4	+	*

+ indicates a functional combination

*indicates that the combination weakly cuts the HIV1_1.2 target.

5

Table VI: Cleavage of the HIV1_1 target by the heterodimeric variants

HIV1_1 target		Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77 of the I-CreI variants cleaving the HIV1_1.4 target (ex: KRSRES/TYSNI stands for K28, R30, S32, R33, E38, S40/ T44, Y68, S70, N75 and I77)	
		KNSTQK/RYS DN SEQ ID NO:11	KCSTQR/RYS DQ SEQ ID NO:10
Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77 Of I-CreI variants cleaving the HIV1_1.3 target (ex: KRGYQS/RHRDI stands for K28, R30, G32, Y33, Q38, S40/ R44, H68, R70, D75 and I77)	KGSYRS/NYSRQ SEQ ID NO:5		
	KGSYRS/NYSRY+162P SEQ ID NO:13		
	KGSYRS/NYSRY SEQ ID NO:2	*	
	KGSYRS/NYSRI SEQ ID NO:1		
	KGSYRS/VERNR SEQ ID NO:3		
	KGSYRS/IERNR SEQ ID NO:4		

+ indicates a functional combination

*indicates that the combination weakly cuts the HIV1_1 target.

10

Example 5: Improvement of meganucleases cleaving HIV1_1 by random mutagenesis of proteins cleaving HIV1_1.3 and assembly with proteins cleaving HIV1_1.4

I-CreI variants able to cleave the HIV1_1.2 and HIV1_1 target by assembly of variants cleaving the palindromic HIV1_1.3 and HIV1_1.4 target have been previously identified in example 4. However, these variants display stronger activity with the HIV1_1.2 target compared to the HIV1_1 target.

Therefore six variants cleaving HIV1_1.3 were mutagenized, and variants were screened for cleavage activity of HIV1_1.3 and HIV1_1.5 targets. Additionally the mutants with the strongest activity were screened for cleavage activity of HIV1_1 when co-expressed with a variant cleaving HIV1_1.4. According to the structure of the I-CreI protein bound to its target, there is no contact between the 4 central base pairs (positions -2 to 2) and the I-CreI protein (Chevalier *et al.*, Nat. Struct. Biol., 2001, 8, 312-316; Chevalier and Stoddard, Nucleic Acids Res., 2001, 29, 3757-3774; Chevalier *et al.*, J. Mol. Biol., 2003, 329, 253-269). Thus, it is difficult to rationally choose a set of positions to mutagenize, and mutagenesis was performed on the whole protein. Random mutagenesis results in high complexity libraries. Therefore, to limit the complexity of the variant libraries to be tested, only one of the two components of the heterodimers cleaving HIV1_1 was mutagenized.

Thus, in a first step, proteins cleaving HIV1_1.3 were mutagenized and their homodimeric cleavage activity was determined, and in a second step, it was assessed whether they could cleave HIV1_1 when co-expressed with a protein cleaving HIV1_1.4.

A) Material and Methods

a) Construction of libraries by random mutagenesis

Random mutagenesis was performed on a pool of chosen variants, by PCR using Mn^{2+} . PCR reactions were carried out that amplify the I-CreI coding sequence using the primers preATGCreFor (5'-gcataaattactatacttctatagacacgcaaacacaaatacacagcgcccttgccacc-3'; SEQ ID NO: 24) and ICReIpostRev (5'-ggctcgaggagctcgtctagaggatcgctcgagttatcagtcggccgc-3'; SEQ ID NO: 25), which are common to the pCLS0542 (Figure 9) and pCLS1107 (Figure 11) vectors. Approximately 25 ng of the PCR product and 75 ng of vector DNA

(pCLS0542) linearized by digestion with *NcoI* and *EagI* were used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (*MAT α* , *trp1* Δ 63, *leu2* Δ 1, *his3* Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). Expression plasmids containing an intact
5 coding sequence for the I-CreI variant were generated by *in vivo* homologous recombination in yeast.

b) Mating of meganuclease expressing clones and screening in yeast

Mating was performed as previously described in example 2. Positive resulting clones were verified by sequencing (MILLEGEN) as described in
10 example 2.

c) Variant-target yeast strains, screening and sequencing

The yeast strain FYBL2-7B (*MAT α* , *ura3* Δ 851, *trp1* Δ 63, *leu2* Δ 1, *lys2* Δ 202) containing the HIV1_1 target in the yeast reporter vector (pCLS1055, Figure 8) was transformed with one variant, in the kanamycin vector (pCLS1107),
15 cutting the HIV1_1.4 target, using a high efficiency LiAc transformation protocol. Variant-target yeast strains were used as target strains for mating assays as described in example 4. Positives resulting clones were verified by sequencing (MILLEGEN) as described in example 2.

B) Results

20 Six variants cleaving HIV1_1.3, were pooled, randomly mutagenized and transformed into yeast. The sequences of the variants subjected to random mutagenesis are described in table VII.

2232 transformed clones were screened for cleavage against the HIV1_1.3 and HIV1_1.5 DNA targets. A total of 297 positive clones were found to
25 cleave HIV1_1.3, while only 6 of those cleaved the HIV1_1.5 target. Sequencing of the 93 clones showing the strongest activity allowed the identification of 51 novel endonuclease variants. An example of the identified variants is presented in table VIII and in figure 14.

Table VII: Sequences corresponding to the variants cleaving the HIV1_1.3 DNA target used for improvement by random mutagenesis

Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77 of I-CreI variants cleaving the HIV1_1.3 target (ex: KRGYQS/RHRDI stands for K28, R30, G32, Y33, Q38, S40/ R44, H68, R70, D75 and I77)	
KGSYRS/NYSRQ	SEQ ID NO:5
KGSYRS/NYSRY +162P	SEQ ID NO:13
KGSYRS/NYSRY	SEQ ID NO:2
KGSYRS/NYSRI	SEQ ID NO:1
KGSYRS/VERNR	SEQ ID NO:3
KGSYRS/IERNR	SEQ ID NO:4

5 **Table VIII: Examples of 10 functional variants displaying strong cleavage activity for HIV1_1.3.**

Optimized variants HIV1_1.3	
SEQ ID NO: 26	<i>I-CreI</i> 30G 38R 44V 54L 68E 75N 77R 80K 81T 132V 163R
SEQ ID NO: 27	<i>I-CreI</i> 30G 38R 44V 54L 68E 75N 77R 80K 99R 111H
SEQ ID NO: 28	<i>I-CreI</i> 30G 38R 44N 68Y 70S 75R 77Y 79N
SEQ ID NO: 29	<i>I-CreI</i> 30G 38R 44V 54L 68Y 70S 75R 77Y 100N
SEQ ID NO: 30	<i>I-CreI</i> 30G 38R 44N 68Y 70S 75R 77Y 162P
SEQ ID NO: 31	<i>I-CreI</i> 30G 38R 44N 68Y 70S 75R 77Y 154R
SEQ ID NO: 32	<i>I-CreI</i> 30G 38R 44N 57R 68Y 70S 75R 77Y
SEQ ID NO: 33	<i>I-CreI</i> 30G 38R 44N 50R 64A 68Y 70S 75R 93Q
SEQ ID NO: 34	<i>I-CreI</i> 30G 38R 44N 68Y 70S 75R 77Y 159R
SEQ ID NO: 35	<i>I-CreI</i> 30G 38R 44N 68Y 70S 75R 77Y 107R 162P

* Mutations resulting from random mutagenesis are in bold.

10 The 93 clones showing the highest cleavage activity on target HIV1_1.3 were then mated with a yeast strain that contains (i) the HIV1_1 target in a reporter plasmid (ii) an expression plasmid containing a variant that cleaves the HIV1_1.4 target (*I-CreI* 33T,40K,44R,68Y,70S,77N +132V or KNSTQK/RYS DN +132V, according to the nomenclature of Table I). After mating with this yeast strain, 41 clones were found to cleave the HIV1_1 target more efficiently than the original variant. Thus, 41
15 positives contained proteins able to form heterodimers with KNSTQK/RYS DN +132V (SEQ ID NO: 46), that showed cleavage activity on the HIV1_1 target. An

example of positive clones is shown in Figure 15. Sequencing of these 41 positive clones indicates that 31 distinct variants were identified. Ten of these 31 variants are presented as an example in Table VIII.

Example 5bis: Improvement of meganucleases cleaving HIV1_1 by a second round of random mutagenesis of proteins cleaving HIV1_1.3 and assembly with proteins cleaving HIV1_1.4

In order to further improve the activity of the obtained meganucleases, a second round of random mutagenesis was carried out following the same rationale of example 5. For this purpose, four variants cleaving HIV1_1.3 were mutagenized, and variants were screened for cleavage activity of HIV1_1.3 and HIV1_1.5 targets. Additionally the mutants with the strongest activity were screened for cleavage activity of HIV1_1 when co-expressed with a variant cleaving HIV1_1.4.

The materials and methods have previously been described in example 5.

A) Results

Four variants cleaving HIV1_1.3, were pooled, randomly mutagenized and transformed into yeast. The four variants submitted to random mutagenesis correspond to variants described in Table VIII (SEQ ID NO: 26, 27, 28 and 29).

2232 transformed clones were screened for cleavage against the HIV1_1.3 and HIV1_1.5 DNA targets. A total of 79 positive clones were found to cleave HIV1_1.3, while 60 of those cleaved also the HIV1_1.5 target. Sequencing of the 79 clones allowed the identification of 47 novel endonuclease variants. An example of the identified variants is presented in table IX and figure 16.

The 79 clones showing cleaving target HIV1_1.3 were then mated with a yeast strain that contains (i) the HIV1_1 target in a reporter plasmid (ii) an expression plasmid containing a variant that cleaves the HIV1_1.4 target (I-CreI 33T,40K,44R,68Y,70S,77N,132V or KNSTQK/RYS DN +132V, according to the nomenclature of Table I). After mating with this yeast strain, 76 clones were found to cleave the HIV1_1 target. Thus, 76 positives contained proteins able to form heterodimers with KNSTQK/RYS DN +132V (SEQ ID NO: 46) showing cleavage activity on the HIV1_1 target. An example of positives is shown in Figure 17. Sequencing of these 76 positive clones indicates that 44 distinct variants were identified. Ten of these 44 variants are presented as an example in Table IX.

Table IX: Examples of 10 functional variants displaying strong cleavage activity for HIV1_1.3.

Optimized variants HIV1_1.3 (2nd round)	
SEQ ID NO: 36	<i>I-CreI</i> 30G 38R 44V 54L 68E 75N 77R 80K 85Y 99R 111H
SEQ ID NO: 37	<i>I-CreI</i> 2S 30G 38R 44V 54L 68E 75N 77R 80K 89A 99R 111H 132V 155Q 163R
SEQ ID NO: 38	<i>I-CreI</i> 16L 30G 31R 38R 44V 54L 57E 61G 68E 75N 77R 80K 81T 132V 162P
SEQ ID NO: 39	<i>I-CreI</i> 30G 38R 44V 54L 68E 75N 77R 80K 99R 111H 163R
SEQ ID NO: 40	<i>I-CreI</i> 17A 30G 38R 42A 44V 54L 64A 68E 75N 77R 80R 86D 99R 111H
SEQ ID NO: 41	<i>I-CreI</i> 30G 38R 44V 54L 68E 75N 77R 80K 81T 121R 132V 160E
SEQ ID NO: 42	<i>I-CreI</i> 28R 30G 38R 39I 44V 54L 68E 75N 77R 80K 81T 86S 132V 150T 162P
SEQ ID NO: 43	<i>I-CreI</i> 30G 34R 38R 44V 54L 68E 75N 77R 80K 81T 132V 163R
SEQ ID NO: 44	<i>I-CreI</i> 30G 38R 44N 68Y 70S 75R 77Y 79N 100E 105A
SEQ ID NO: 45	<i>I-CreI</i> 16L 30G 38R 44V 54L 68E 75N 77R 80K 99R 111H 112Q

* Mutations resulting from random mutagenesis are in bold.

5

Example 6: Improvement of meganucleases cleaving HIV1_1 by site-directed mutagenesis of proteins cleaving HIV1_1.3 and assembly with proteins cleaving HIV1_1.4

The *I-CreI* variants cleaving HIV1_1.3 described in Table IX issued from random mutagenesis in examples 5 and 5bis were also mutagenized by introducing selected amino-acid substitutions in the proteins and screening for more efficient variants cleaving HIV1_1 in combination with a variant cleaving HIV1_1.4.

Six amino-acid substitutions have been found in previous studies to enhance the activity of *I-CreI* derivatives: these mutations correspond to the replacement of Glycine 19 with Serine (G19S), Phenylalanine 54 with Leucine (F54L), Glutamic acid 80 with Lysine (E80K), Phenylalanine 87 with Leucine (F87L), Valine 105 with Alanine (V105A) and Isoleucine 132 with Valine (I132V). These mutations were introduced into the coding sequence of proteins cleaving HIV1_1.3, and the resulting proteins were tested for their ability to induce cleavage of the HIV1_1 target, upon co-expression with a variant cleaving HIV1_1.4, as well as for the ability to cleave targets HIV1_1.3 and HIV1_1.5.

A) Material and Methods

a) Site-directed mutagenesis

Site-directed mutagenesis libraries were created by PCR on a pool of chosen variants. For example, to introduce the G19S substitution into the coding sequence of the variants, two separate overlapping PCR reactions were carried out that amplify the 5' end (residues 1-24) or the 3' end (residues 14-167) of the I-*CreI* coding sequence. For both the 5' and 3' end, PCR amplification is carried out using a primer with homology to the vector (Gal10F 5'-gcaacttagtgctgacacatacagg-3' (SEQ ID NO: 16) or Gal10R 5'-acaaccttgattggagacttgacc-3' (SEQ ID NO: 17)) and a primer specific to the I-*CreI* coding sequence for amino acids 14-24 that contains the substitution mutation G19S (G19SF 5'-gccggcttggactctgacggtacatcatc-3' (SEQ ID NO: 47) or G19SR 5'-gatgatgctaccgtcagagtccacaaagccggc-3' (SEQ ID NO: 48)).

The same strategy is used with the following pair of oligonucleotides to introduce the mutations leading to the F54L, E80K, F87L, V105A and I132V substitutions in the coding sequences of the variants, respectively:

* F54LF: 5'-accagcgccgttggtgctggacaaactagtg-3' and F54LR: 5'-cactagttgtccagcagccaacggcgctgggt-3' (SEQ ID NO: 49 and 50);

* E80KF: 5'-ttaagcaaatcaagccgtgcacaacttcctg-3' and E80KR: 5'-caggaagtgtgcagcggcttgatttgcctaa-3' SEQ ID NO: 51 and 52);

* F87LF: 5'-aagccgctgcacaacctgctgactcaactgcag-3' and F87LR: 5'-ctgcagttgagtcagcaggtgtgcagcggtt-3' SEQ ID NO: 53 and 54);

* V105AF: 5'-aacaggcaaacctggctctgaaaattatcgaa-3' and V105AR: 5'-ttcgataatttcagagccaggttgcctgtt-3' SEQ ID NO: 55 and 56);

* I132VF: 5'-acctgggtggatcaggttgacgtctgaacgat-3' and I132VR: 5'-atcgttcagagctgcaacctgatccaccaggt-3' SEQ ID NO: 57 and 58).

For each substitution to be introduced, the resulting PCR products contain 33bp of homology with each other. The PCR fragments were purified. The ten PCR fragments were pooled in equimolar amounts to generate a mix containing 50ng of PCR DNA and 75ng of vector DNA (pCLS0542, Figure 9), linearized by digestion with *NcoI* and *EagI*. This mix was used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (*MAT α* , *trp1*Δ63, *leu2*Δ1, *his3*Δ200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002,

350, 87-96). Intact coding sequences containing the substitutions are generated *in vivo* by homologous recombination in yeast.

c) Mating of meganuclease expressing clones and screening in yeast

The experimental procedure is as described in example 5.

5 d) Sequencing of variants

The experimental procedure is as described in example 2.

B) Results

A library containing a population harboring the six amino-acid substitutions (Glycine 19 with Serine, Phenylalanine 54 with Leucine, Glutamic acid
10 80 with Lysine, Phenylalanine 87 with Leucine, Valine 105 with Alanine and Isoleucine 132 with Valine) was constructed on a pool of five variants cleaving HIV1_1.3 (described in Table X). 558 transformed clones were screened for cleavage against the HIV1_1.3 and HIV1_1.5 DNA targets. A total of 395 positive clones were found to cleave HIV1_1.3, while 349 of those cleaved also the HIV1_1.5 target. An
15 example of positive variants is shown in figure 18

The 558 transformed clones were also mated with a yeast strain that contains (i) the HIV1_1 target in a reporter plasmid (ii) an expression plasmid containing a variant that cleaves the HIV1_1.4 target (I-CreI 33T,40K,44R,68Y,70S,77N +132V or KNSTQK/RYSND +132V, according to the
20 nomenclature of Table I). After mating with this yeast strain, 458 clones were found to cleave the HIV1_1. Thus, 458 positives contained proteins able to form heterodimers with KNSTQK/RYSND +132V (SEQ ID NO: 46) showing cleavage activity on the HIV1_1 target. An example of positives is shown in Figure 19.

Sequencing of the 186 clones with the highest cleavage activity on
25 the HIV1_1 target allowed the identification of 138 different endonuclease variants.

The sequence of the five best I-CreI variants cleaving the HIV1_1 target when forming a heterodimer with the KNSTQK/RYSND +132V variant are listed in Table XI.

Table X: Sequences corresponding to the variants cleaving the HIV1_1.3 DNA target used for improvement by site-directed mutagenesis

I-CreI variants cleaving the HIV1_1.3 target*		
SEQ ID NO:	Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77	Unique mutations, compared to the I-CreI sequence
26	KGSYRS/VERNR	30G38R44V54L68E75N77R80K81T132V163R
36	KGSYRS/VERNR	30G38R44V54L68E75N77R80K85Y99R111H
38	KGSYRS/VERNR	16L30G31R38R44V54L57E61G68E75N77R80K81T132V162P
40	KGSYRS/VERNR	17A30G38R42A44V54L64A68E75N77R80R86D99R111H
42	KGSYRS/VERNR	28R30G38R39I44V54L68E75N77R80K81T86S132V150T162P

* The nomenclature of the mutants is the same as for Table I. (ex: KRGYQS/RHRDI stands for K28, R30, G32, Y33, Q38, S40/ R44, H68, R70, D75 and I77)

5

Table XI: Functional variant combinations displaying strong cleavage activity for HIV1_1.

		Optimized* Variants HIV1_1.3 (SEQ ID NO: 59 to 63)
VARIANT HIV1_1.4 (SEQ ID NO: 46)	I-CreI 28K30N32S33T38Q40S44V 68E70R75N77R132V KNSTQK/RYSND +132V	I-CreI 19S 30G 38R 44V 54L 68E 75N 77R 80K 81T 132V
		I-CreI 19S 30G 38R 44V 54L 68E 75N 77R 80K 81T 86D 99R 111H 132V 162P
		I-CreI 19S 30G 31R 38R 44V 54L 64A 68E 75N 77R 80R 86D 105A 132V
		I-CreI 19S 30G 38R 44V 54L 68E 75N 77R 80K 85Y 99R 111H 162P
		I-CreI 19S 30G 38R 44V 54L 64A 68E 75N 77R 80R 86D 99R 111H

* Mutations resulting from site-directed mutagenesis are in bold.

Example 7: Improvement of meganucleases cleaving HIV1_1 by random mutagenesis of proteins cleaving HIV1_1.4 and assembly with proteins cleaving HIV1_1.3

As a complement to example 4 we also decided to perform random mutagenesis with variants that cleave HIV1_1.4. The mutagenized proteins cleaving HIV1_1.4 were then tested to determine if they could efficiently cleave HIV1_1 when co-expressed with a protein cleaving HIV1_1.3.

15

A) Material and Methods

a) Construction of libraries by random mutagenesis

Random mutagenesis was performed on a pool of chosen variants, by PCR using Mn^{2+} . PCR reactions were carried out that amplify the I-CreI coding sequence using the primers preATGCreFor (5'-gcataaattactatacttctatagacacgcaaacacaaatacacagcgcccttgccacc-3'; SEQ ID NO: 24) and ICReIpostRev (5'-ggctcgaggagctcgtctagaggatcgctcgagttatcagtcggccgc-3'; SEQ ID NO: 25). Approximately 25 ng of the PCR product and 75 ng of vector DNA (pCLS1107, Figure 11) linearized by digestion with *Dra*III and *Ngo*MIV were used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (*MAT* α , *trp1* Δ 63, *leu2* Δ 1, *his3* Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). Expression plasmids containing an intact coding sequence for the I-CreI variant were generated by *in vivo* homologous recombination in yeast.

15 b) Variant-target yeast strains, screening and sequencing

The yeast strain FYBL2-7B (*MAT* *a*, *ura3* Δ 851, *trp1* Δ 63, *leu2* Δ 1, *lys2* Δ 202) containing the HIV1_1 target in the yeast reporter vector (pCLS1055, Figure 8) was transformed with variants, in the leucine vector (pCLS0542), cutting the HIV1_1.3 target, using a high efficiency LiAc transformation protocol. Variant-target yeast strains were used as target strains for mating assays as described in example 4. Positives resulting clones were verified by sequencing (MILLEGEN) as described in example 2.

B) Results

Six variants cleaving HIV1_1.4 were pooled, randomly mutagenized and transformed into yeast. The sequences of the variants subjected to random mutagenesis are described in table XII.

2232 transformed clones were screened for cleavage against the HIV1_1.4 and HIV1_1.6 DNA targets. A total of 388 positive clones were found to cleave HIV1_1.4, while 88 of those also cleaved the HIV1_1.6 target. Sequencing of the 89 clones showing the strongest activity allowed the identification of 50 novel endonuclease variants. An example of the identified variants is presented in table XIII and in figure 20.

Table XII: Sequences corresponding to the variants cleaving the HIV1_1.4 DNA target used for improvement by random mutagenesis

Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77 of I-CreI variants cleaving the HIV1_1.4 target (ex: KRGYQS/RHRDI stands for K28, R30, G32, Y33, Q38, S40/ R44, H68, R70, D75 and I77)	
QNSSRK/KYSES	SEQ ID NO:7
KNSCAS/KYSES	SEQ ID NO:8
KNSSRN/KYSES	SEQ ID NO:9
KCSTQR/RYSdq	SEQ ID NO:10
KNSTQK/RYSdN	SEQ ID NO:11
KNSCAS/RYSdN	SEQ ID NO:66

5 **Table XIII: Examples of 10 functional variants displaying strong cleavage activity for HIV1_1.4.**

Optimized variants HIV1_1.4	
SEQ ID NO: 46	I-CreI 33T 40K 44R 68Y 70S 77N 132V
SEQ ID NO: 67	I-CreI 33T 40K 44R 68C 70S 77N 160R
SEQ ID NO: 68	I-CreI 33T 40K 44R 68Y 70S 77N
SEQ ID NO: 69	I-CreI 33T 40K 44R 68Y 70S 77N 157K
SEQ ID NO: 70	I-CreI 16L 33T 40R 44R 68Y 70S 77N
SEQ ID NO: 71	I-CreI 33T 40K 43L 44R 68Y 70S 77N
SEQ ID NO: 72	I-CreI 6D 33T 40K 44R 68Y 70S 75E 77S 83S 154N 161P
SEQ ID NO: 73	I-CreI 38R 44R 70S 75N 105A 131R
SEQ ID NO: 74	I-CreI 7E 33T 40K 44K 68Y 70S 72P 75E 77S 83S
SEQ ID NO: 75	I-CreI 3A 33C 40K 44R 68Y 70S 77N 129A 151G

* Mutations resulting from random mutagenesis are in bold.

10 The 89 clones showing the highest cleavage activity on target HIV1_1.4 were then mated with a yeast strain that contains (i) the HIV1_1 target in a reporter plasmid (ii) an expression plasmid containing a variant that cleaves the HIV1_1.3 target (I-CreI 30G,38R,44V,68E,75N,77R,54L,80K,81T,132V,163R or KGSYRS/VERN

15 46 positives contained proteins able to form heterodimers with KGSYRS/VERN +54L+80K+81T+132V+163R (SEQ ID NO: 26), that showed cleavage activity on the

HIV1_1 target. An example of positives is shown in Figure 21. Sequencing of these 88 positive clones indicates that 46 distinct variants were identified. Ten of these 46 variants are presented as an example in Table XIII.

Example 7bis: Improvement of meganucleases cleaving HIV1_1 by a second round of random mutagenesis of proteins cleaving HIV1_1.4 and assembly with proteins cleaving HIV1_1.3

In order to further improve the activity of the obtained meganucleases, a second round of random mutagenesis was carried out following the same rationale of example 7. For this purpose, four variants cleaving HIV1_1.4 were mutagenized, and variants were screened for cleavage activity of HIV1_1.4 and HIV1_1.6 targets. Additionally the mutants with the strongest activity were screened for cleavage activity of HIV1_1 when co-expressed with a variant cleaving HIV1_1.3.

The materials and methods have previously been described in example 7.

A) Results

Four variants cleaving HIV1_1.4, were pooled, randomly mutagenized and transformed into yeast. The four variants submitted to random mutagenesis correspond to variants described in Table XIII (SEQ ID NO: 46, 68, 69 and 71).

2232 transformed clones were screened for cleavage against the HIV1_1.4 and HIV1_1.6 DNA targets. A total of 59 positive clones were found to cleave HIV1_1.4, while 16 of those cleaved also the HIV1_1.6 target. Sequencing of the 49 clones allowed the identification of 35 novel endonuclease variants. An example of the identified variants is presented in table XIV and figure 22.

The 59 clones showing cleaving target HIV1_1.4 were then mated with a yeast strain that contains (i) the HIV1_1 target in a reporter plasmid (ii) an expression plasmid containing a variant that cleaves the HIV1_1.3 target (I-CreI 30G,38R,44N,68Y,70S,75R,77Y +79N or KGSYRS/NYSRY +79N, according to the nomenclature of Table I). After mating with this yeast strain, 42 clones were found to cleave the HIV1_1. Thus, 42 positives contained proteins able to form heterodimers with KGSYRS/NYSRY +79N (SEQ ID NO: 28) showing cleavage activity on the HIV1_1 target. An example of positives is shown in Figure 23. Sequencing of these

42 positive clones indicates that 35 distinct variants were identified. Ten of these 35 variants are presented as an example in Table XIV.

Table XIV: Examples of 10 functional variants displaying strong cleavage activity for HIV1_1.4.

5

Optimized variants HIV1_1.4 (2nd round)	
SEQ ID NO: 76	<i>I-CreI</i> 33T 40K 44R 68Y 70S 77N 157K
SEQ ID NO: 77	<i>I-CreI</i> 33T 40K 44R 68Y 70S 77N 117G
SEQ ID NO: 78	<i>I-CreI</i> 6D 33T 40K 44R 68Y 70S 77N
SEQ ID NO: 79	<i>I-CreI</i> 33T 40K 44R 68Y 70S 77N
SEQ ID NO: 80	<i>I-CreI</i> 8V 33T 40K 44R 64A 68Y 70S 77N 103K 116R 132V
SEQ ID NO: 81	<i>I-CreI</i> 16L 33T 40K 43L 44R 68Y 70S 77N
SEQ ID NO: 82	<i>I-CreI</i> 33T 40K 44R 57E 68N 70S 77N 121R 132V
SEQ ID NO: 83	<i>I-CreI</i> 33T 40K 44R 64A 68Y 70T 77N 153G
SEQ ID NO: 84	<i>I-CreI</i> 33T 40K 44R 68Y 70S 77N 105L 156N 157K
SEQ ID NO: 85	<i>I-CreI</i> 33T 40K 43L 44R 68Y 70S 77N 105A

* Mutations resulting from random mutagenesis are in bold.

Example 8: Strategy for engineering meganucleases cleaving the HIV1_3 target from the HIV1 virus

10

The HIV1_3 target is a 22 bp (non-palindromic) target located in U5 region of the proviral LTRs. Since the LTRs are duplicated sequences flanking the viral ORFs in the integrated provirus, the HIV1_3 target is present twice in the HIV1 provirus. This target is precisely located at positions 599-620 and 9674-9695 of the HIV-1 pNL4-3 vector (accession number AF324493, Adachi et al., J. Virol., 1986, 59, 284-291), a subtype B infectious molecular clone.

15

The HIV1_3 sequence (SEQ ID NO: 325) is partly a patchwork of the 10CAG_P, 10ACA_P, 5CCT_P and 5_GAC_P targets (Figure 24) which are cleaved by previously identified meganucleases, obtained as described in International PCT Applications WO 2006/097784 and WO 2006/097853; Arnould et al., J. Mol. Biol., 2006, 355, 443-458; Smith et al., Nucleic Acids Res., 2006. Thus, HIV1_3 could be cleaved by combinatorial variants resulting from these previously identified meganucleases.

20

25

The 10CAG_P, 10ACA_P, 5CCT_P and 5_GAC_P target sequences are 24 bp derivatives of C1221, a palindromic sequence cleaved by *I-CreI* (Arnould et al., precited). However, the structure of *I-CreI* bound to its DNA target suggests that the two external base pairs of these targets (positions -12 and 12) have no impact on

binding and cleavage (Chevalier et al., Nat. Struct. Biol., 2001, 8, 312-316; Chevalier and Stoddard, Nucleic Acids Res., 2001, 29, 3757-3774; Chevalier et al., J. Mol. Biol., 2003, 329, 253-269), and in this study, only positions -11 to 11 were considered. Consequently, the HIV1_3 series of targets were defined as 22 bp sequences instead of 24 bp. HIV1_3 differs from C1221 in the 4 bp central region. According to the structure of the I-CreI protein bound to its target, there is no contact between the 4 central base pairs (positions -2 to 2) and the I-CreI protein (Chevalier et al., Nat. Struct. Biol., 2001, 8, 312-316; Chevalier and Stoddard, Nucleic Acids Res., 2001, 29, 3757-3774; Chevalier et al., J. Mol. Biol., 2003, 329, 253-269). Thus, the bases at these positions should not impact the binding efficiency. However, they could affect cleavage, which results from two nicks at the edge of this region. Thus, the TTTA sequence in -2 to 2 was first substituted with the GTAC sequence from C1221, resulting in target HIV1_3.2 (SEQ ID NO: 326, Figure 24). Then, two palindromic targets, HIV1_3.3 (SEQ ID NO: 327) and HIV1_3.4 (SEQ ID NO: 328), were derived from HIV1_3.2 (Figure 24). Since HIV1_3.3 and HIV1_3.4 are palindromic, they should be cleaved by homodimeric proteins. Two other pseudo-palindromic targets were derived from these two, containing the TTTA sequence in -2 to 2 (targets HIV1_3.5 (SEQ ID NO: 329) and HIV1_3.6 (SEQ ID NO: 330), figure 24). Thus, proteins able to cleave HIV1_3.3 and HIV1_3.4 targets or, preferentially, the pseudo-palindromic targets as homodimers were first designed (examples 9 and 10) and then co-expressed to obtain heterodimers cleaving HIV1_3 (example 11). Heterodimers cleaving the HIV1_3.2 or HIV1_3 targets could not be identified. In order to obtain cleavage activity for the HIV1_3 target, a series of variants cleaving HIV1_3.3 and HIV1_3.4 was chosen, and then refined. The chosen variants were subjected to random or site-directed mutagenesis, and used to form novel heterodimers that were screened against the HIV1_3 target (examples 12, 13, 14 and 15). Heterodimers could be identified with an improved cleavage activity for the HIV1_3 target.

Example 9: Identification of meganucleases cleaving HIV1_3.3

This example shows that I-CreI variants can cut the HIV1_3.3 target sequence derived from the left part of the HIV1_3.2 target in a palindromic form (Figure 24).

HIV1_3.3 is similar to 10CAG_P at positions ± 1 , ± 2 , ± 6 , ± 8 , ± 9 , and ± 10 and to 5CCT_P at positions ± 1 , ± 2 , ± 3 , ± 4 , ± 5 and ± 6 . It was hypothesized that positions ± 7 and ± 11 would have little effect on the binding and cleavage activity. Variants able to cleave the 10CAG_P target were obtained by mutagenesis of I-CreI
 5 N75 or D75, at positions 28, 30, 32, 33, 38, 40 and 70, as described previously in Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156. Variants able to cleave 5CCT_P were obtained by mutagenesis on I-CreI N75 at positions 24, 44, 68, 70, 75 and 77 as described in Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; Smith *et al.* Nucleic Acids Res.,
 10 2006, 34, e149; International PCT Applications WO 2006/097784, WO 2006/097853, WO 2007/060495 and WO 2007/049156.

Both sets of proteins are mutated at position 70. However, the existence of two separable functional subdomains was hypothesized. This implies that this position has little impact on the specificity at bases 10 to 8 of the target.
 15 Mutations at positions 24 found in variants cleaving the 5CCT_P target will be lost during the combinatorial process. But it was hypothesized that this will have little impact on the capacity of the combined variants to cleave the HIV1_3.3 target.

Therefore, to check whether combined variants could cleave the HIV1_3.3 target, mutations at positions 44, 68, 70, 75 and 77 from proteins cleaving 5CCT_P
 20 were combined with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10CAG_P.

A) Material and Methods

a) Construction of target vector

The target was cloned as follows: an oligonucleotide corresponding to the HIV1_3.3
 25 target sequence flanked by gateway cloning sequences was ordered from PROLIGO:
 5' TGGCATACAAGTTTCTCAGACCCTGTACAGGGTCTGAGCAATCGTCTGTCA 3'
 (SEQ ID NO: 86). The same procedure was followed for cloning the HIV1_1.5 target, using the oligonucleotide:
 5' TGGCATACAAGTTTCTCAGACCCTTTTAAGGGTCTGAGCAATCGTCTGTCA 3'
 30 (SEQ ID NO: 87). Double-stranded target DNA, generated by PCR amplification of the single stranded oligonucleotide, was cloned using the Gateway protocol (INVITROGEN) into the yeast reporter vector (pCLS1055, Figure 8). Yeast reporter

vector was transformed into *Saccharomyces cerevisiae* strain FYBL2-7B (*MAT a*, *ura3* Δ 851, *trp1* Δ 63, *leu2* Δ 1, *lys2* Δ 202), resulting in a reporter strain.

b) Construction of combinatorial mutants

I-*CreI* variants cleaving 10CAG_P or 5CCT_P were previously
5 identified, as described in Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156, and Arnould
et al., J. Mol. Biol., 2006, 355, 443-458; International PCT Applications WO
2006/097784 and WO 2006/097853, respectively for the 10AGA_P and 5TAC_P
10 targets. In order to generate I-*CreI* derived coding sequences containing mutations
from both series, separate overlapping PCR reactions were carried out that amplify the
5' end (aa positions 1-43) or the 3' end (positions 39-167) of the I-*CreI* coding
sequence. For both the 5' and 3' end, PCR amplification is carried out using primers
(Gal10F 5'-gcaactttagtgctgacacatacagg-3' (SEQ ID NO: 16) or Gal10R 5'-
acaaccttgattggagacttgacc-3' (SEQ ID NO: 17)) specific to the vector (pCLS0542,
15 Figure 9) and primers (assF 5'-ctannnttgacctt-3' (SEQ ID NO: 18) or assR 5'-
aaaggtcaannntag-3' (SEQ ID NO: 19), where nnn codes for residue 40, specific to the
I-*CreI* coding sequence for amino acids 39-43. The PCR fragments resulting from the
amplification reaction realized with the same primers and with the same coding
sequence for residue 40 were pooled. Then, each pool of PCR fragments resulting
20 from the reaction with primers Gal10F and assR or assF and Gal10R was mixed in an
equimolar ratio. Finally, approximately 25 ng of each final pool of the two
overlapping PCR fragments and 75 ng of vector DNA (pCLS0542, Figure 9)
linearized by digestion with *NcoI* and *EagI* were used to transform the yeast
Saccharomyces cerevisiae strain FYC2-6A (*MAT* α , *trp1* Δ 63, *leu2* Δ 1, *his3* Δ 200)
25 using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods
Enzymol., 2002, 350, 87-96). An intact coding sequence containing both groups of
mutations is generated by *in vivo* homologous recombination in yeast.

c) Mating of meganuclease expressing clones and screening in yeast

Screening was performed as described previously (Arnould *et al.*, J.
30 Mol. Biol., 2006, 355, 443-458). Mating was performed using a colony gridded
(QpixII, GENETIX). Variants were gridded on nylon filters covering YPD plates,
using a low gridding density (4-6 spots/cm²). A second gridding process was

performed on the same filters to spot a second layer consisting of the reporter-harboring yeast strain. Membranes were placed on solid agar YPD rich medium, and incubated at 30 °C for one night, to allow mating. Next, filters were transferred to synthetic medium, lacking leucine and tryptophan, with galactose (2 %) as a carbon source, and incubated for five days at 37 °C, to select for diploids carrying the expression and target vectors. After 5 days, filters were placed on solid agarose medium with 0.02 % X-Gal in 0.5 M sodium phosphate buffer, pH 7.0, 0.1 % SDS, 6% dimethyl formamide (DMF), 7 mM β -mercaptoethanol, 1% agarose, and incubated at 37°C, to monitor β -galactosidase activity. Results were analyzed by scanning and quantification was performed using appropriate software.

d) Sequencing of variants

To recover the variant expression plasmids, yeast DNA was extracted using standard protocols and used to transform *E. coli*. Sequencing of variant ORFs was then performed on the plasmids by MILLEGEN SA. Alternatively, ORFs were amplified from yeast DNA by PCR (Akada et al., Biotechniques, 2000, 28, 668-670), and sequencing was performed directly on the PCR product by MILLEGEN SA.

B) Results

I-CreI combinatorial variants were constructed by associating mutations at positions 44, 68, 70, 75 and 77 from proteins cleaving 5CCT_P with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10CAG_P on the I-CreI scaffold, resulting in a library of complexity 1600. Examples of combinatorial variants are displayed in Table XV. This library was transformed into yeast and 3348 clones (2 times the diversity) were screened for cleavage against the HIV1_3.3 and HIV1_3.5 DNA targets. 10 positive clones were found to cleave the HIV1_3.3 target, which after sequencing turned out to correspond to 7 different novel endonuclease variants (Table XVI). These variants showed no cleavage activity of the HIV1_3.5 DNA target. Examples of positives are shown in Figure 25. Some of the variants obtained display non parental combinations at positions 28, 30, 32, 33, 38, 40 or 44, 68, 70, 75, 77 (SEQ ID NO: 92 to 94, Table XVI). Such combinations likely result from PCR artifacts during the combinatorial process. Alternatively, the variants may be I-CreI combined variants

resulting from micro-recombination between two original variants during *in vivo* homologous recombination in yeast.

Table XV: Panel of variants* theoretically present in the combinatorial library

Amino acids at positions 44, 68, 70, 75 and 77 (ex : ARNNI stands for A44, R68, N70, N75 and I77)	Amino acids at positions 28, 30, 32, 33, 38 and 40 (ex: KHSSQS stands for K28, H30, S32, S33, Q38 and S40)										
	KQDYQS	KYTCQS	KCSCQS	KDSRQS	KTSCYS	KNTCQS	KDSRSS	KNSNYR	KGSYQG	KSSQQS	KTEYQS
KNSNI											
KTGNI											
KRTNI						+					
KGTNI											
DASKR											
KASNI											
KTSDR											
KYSDY											
RYSNN											
KYSYN											
KESDR											
KASDK			+								
RTSNN											
QHHNI											
TRSRT											
KSNDI								+			
KDSNR											
KESDK											
PCSYT											
KESNR			+								
RYSNI											
KNTNI											
KTSDI											
RRSND											

5

*Only 264 out of the 1600 combinations are displayed.

+ indicates that a functional combinatorial variant cleaving the HIV1_3.3 target was found among the identified positives.

Table XVI: I-CreI variants capable of cleaving the HIV1_3.3 DNA target.

Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77 of the I-CreI variants (ex: KRSRES/TYSNI stands for K28, R30, S32, R33, E38, S40/ T44, Y68, S70, N75 and I77)	SEQ ID NO:
KNSNYR/KSNDI	88
KCSCQS/KASDK	89
KCSCQS/KESNR	90
KNTCQS/KRTNI	91
KNKCQS/QEGNL	92
KNSNYS/KYSYI	93
KSSQQS/QASET	94

Example 10: Making of meganucleases cleaving HIV1_3.4

This example shows that I-CreI variants can cleave the HIV1_3.4 DNA target sequence derived from the right part of the HIV1_3.2 target in a palindromic form (Figure 24).

HIV1_3.4 is similar to 5GAC_P at positions ± 1 , ± 2 , ± 3 , ± 4 , ± 5 and ± 8 and to 10ACA_P at positions ± 1 , ± 2 , ± 3 , ± 4 , ± 8 , ± 9 and ± 10 . It was hypothesized that positions ± 6 , ± 7 and ± 11 would have little effect on the binding and cleavage activity. Variants able to cleave 5GAC_P were obtained by mutagenesis of I-CreI N75 at positions 44, 68, 70, 75 and 77, as described previously (Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2006/097784, WO 2006/097853, WO 2007/060495 and WO 2007/049156). Variants able to cleave the 10ACA_P target were obtained by mutagenesis of I-CreI N75 or D75, at positions 28, 30, 32, 33, 38, 40 and 70, as described previously in Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156.

Both sets of proteins are mutated at position 70. However, the existence of two separable functional subdomains was hypothesized. This implies that this position has little impact on the specificity at bases 10 to 8 of the target.

Therefore, to check whether combined variants could cleave the HIV1_3.4 target, mutations at positions 44, 68, 70, 75 and 77 from proteins cleaving

5GAC_P were combined with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10ACA_P.

A) Material and Methods

a) Construction of target vector

- 5 The experimental procedure is as described in example 2, with the exception that different oligonucleotides corresponding to the HIV1_3.4 and HIV1_3.6 targets. The oligonucleotide used for the HIV1_3.4 target was:

5'

TGGCATAACAAGTTTCCACACTGACGTACGTCAAGTGTGGCAATCGTCTGTCA

- 10 3' (SEQ ID NO: 95), and

5'

TGGCATAACAAGTTTCCACACTGACTTTAGTCAGTGTGGCAATCGTCTGTCA

3' (SEQ ID NO: 96) for HIV1_3.6 target.

b) Construction of combinatorial variants

- 15 I-CreI variants cleaving 10ACA_P or 5GAC_P were previously identified, as described in Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156, and Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; International PCT Applications WO 2006/097784 and WO 2006/097853, respectively for the 10ACA_P and 5GAC_P
- 20 targets. In order to generate I-CreI derived coding sequences containing mutations from both series, separate overlapping PCR reactions were carried out that amplify the 5' end (aa positions 1-43) or the 3' end (positions 39-167) of the I-CreI coding sequence. For both the 5' and 3' end, PCR amplification is carried out using primers (Gal10F 5'-gcaacttagtgctgacacatacagg-3' (SEQ ID NO: 16) or Gal10R 5'-
- 25 acaaccttgattggagacttgacc-3' (SEQ ID NO: 17)) specific to the vector (pCLS1107, Figure 11) and primers (assF 5'-ctannnttgaccttt-3' (SEQ ID NO: 18) or assR 5'-aaaggtcaannntag-3' (SEQ ID NO: 19)), where nnn codes for residue 40, specific to the I-CreI coding sequence for amino acids 39-43. The PCR fragments resulting from the amplification reaction realized with the same primers and with the same coding
- 30 sequence for residue 40 were pooled. Then, each pool of PCR fragments resulting from the reaction with primers Gal10F and assR or assF and Gal10R was mixed in an equimolar ratio. Finally, approximately 25 ng of each final pool of the two

overlapping PCR fragments and 75 ng of vector DNA (pCLS1107, Figure 11) linearized by digestion with DraIII and NgoMIV were used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (MAT α , trp1 Δ 63, leu2 Δ 1, his3 Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods
5 Enzymol., 2002, 350, 87-96). An intact coding sequence containing both groups of mutations is generated by *in vivo* homologous recombination in yeast.

c) Mating of meganuclease expressing clones and screening in yeast

Screening was performed as described previously (Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458). Mating was performed using a colony gridder
10 (QpixII, GENETIX). Variants were gridded on nylon filters covering YPD plates, using a low gridding density (4-6 spots/cm²). A second gridding process was performed on the same filters to spot a second layer consisting of the reporter-harboring yeast strain. Membranes were placed on solid agar YPD rich medium, and incubated at 30 °C for one night, to allow mating. Next, filters were transferred to
15 synthetic medium, lacking tryptophan, adding G418, with galactose (2 %) as a carbon source, and incubated for five days at 37 °C, to select for diploids carrying the expression and target vectors. After 5 days, filters were placed on solid agarose medium with 0.02 % X-Gal in 0.5 M sodium phosphate buffer, pH 7.0, 0.1 % SDS, 6% dimethyl formamide (DMF), 7 mM β -mercaptoethanol, 1% agarose, and
20 incubated at 37°C, to monitor β -galactosidase activity. Results were analyzed by scanning and quantification was performed using appropriate software. Positives resulting clones were verified by sequencing (MILLEGEN) as described in example 2.

B) Results

I-CreI combinatorial variants were constructed by associating muta-
25 tions at positions 44, 68, 70, 75 and 77 from proteins cleaving 5GAC_P with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10ACA_P on the I-CreI scaffold, resulting in a library of complexity 2280. Examples of combinatorial variants are displayed in Table XVII. This library was transformed into yeast and 3348 clones (1.5 times the diversity) were screened for cleavage against the HIV1_3.4 and
30 HIV1_3.6 DNA targets. A total of 305 positive clones were found to cleave HIV1_3.4, and two of those variants showed cleavage activity on the HIV1_3.6 target. DNA Sequencing of these 93 strongest clones allowed the identification of 64 novel

endonuclease variants. Examples of positives are shown in Figure 26. Some variants identified display non parental combinations at positions 28, 30, 32, 33, 38, 40 or 44, 68, 70, 75, 77 as well as additional mutations (see examples Table XVIII, SEQ ID NO: 102 to 104). Such variants likely result from PCR artifacts during the combinatorial process. Alternatively, the variants may be I-CreI combined variants resulting from micro-recombination between two original variants during *in vivo* homologous recombination in yeast.

Table XVII: Panel of variants* theoretically present in the combinatorial library

Amino acids at positions 44, 68, 70, 75 and 77 (ex: ARNNI stands for A44, R68, N70, N75 and I77)	Amino acids at positions 28, 30, 32, 33, 38 and 40 (ex: KHSSQS stands for K28, H30, S32, S33, Q38 and S40)										
	KRSYER	KRSYES	KNSYYS	KSSCQS	KNSRES	KNTYSS	KNDYYS	KNSRER	KRDYQS	KNEYYS	KNTYAS
AYSRI		+	+					+	+		
ARSYT			+						+		
IRRNIR											
NYSRQ		+	+		+			+	+	+	+
YDSRV											
YKSRQ											
NRSRV		+									
YRSYN											
NYSRY		+			+		+	+			
NRSRD									+		
AHRNI											
NRSRN	+	+	+		+				+		
YSSRI		+							+	+	
ARSYQ											
YSSRQ									+		
YSSRV		+	+	+	+	+	+		+	+	
AYSRT		+									
NASRY											
ARSYY											
NKSRN		+									
NTSRQ		+							+		
NRSRQ		+							+		
NYSRD											
NRSRV									+		

10 * Only 264 out of the 2280 combinations are displayed.

+ indicates that a functional combinatorial variant cleaving the HIV1_3.4 target was found among the identified positives.

Table XVIII: I-CreI variants capable of cleaving the HIV1_3.4* DNA target.

Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77 of the I-CreI variants (ex: KRSRES/TYSNI stands for K28, R30, S32, R33, E38, S40/ T44, Y68, S70, N75 and I77)	SEQ ID NO:
KNSYYS/YSSRV +105A	97
KRSYER/NRSRN	98
KRSYES/YSSRQ	99
KNSYYS/YSSRV	100
KNDYYS/YSSRV	101
KRDYQS/YRSRE	102
KRDYYS/NRSRN	103
KNTYRS/YYSRT	104

Example 11: Making of meganucleases cleaving HIV1_3.2 and HIV1_3

5 I-CreI variants able to cleave each of the palindromic HIV1_3.2 derived targets (HIV1_3.3 and HIV1_3.4) were identified in example 9 and example 10. Pairs of such variants (one cutting HIV1_3.3 and one cutting HIV1_3.4) were co-expressed in yeast. Upon co-expression, there should be three active molecular species, two homodimers, and one heterodimer. It was assayed whether the
10 heterodimers that should be formed, cut the HIV1_3.2 and the non palindromic HIV1_3 targets.

A) Materials and Methods**a) Construction of target vector**

The experimental procedure is as described in example 9, with the exception that an
15 oligonucleotide corresponding to the HIV1_3.2 target sequence:

5' TGGCATACAAGTTTCTCAGACCCTGTACGTCAGTGTGGCAATCGTCTGTCA
3'(SEQ ID NO: 317) or the HIV1_3 target sequence:

5' TGGCATACAAGTTTCTCAGACCCTTTTAGTCAGTGTGGCAATCGTCTGTCA 3'
(SEQ ID NO: 318) was used.

b) Co-expression of variants

20 Yeast DNA was extracted from variants cleaving the HIV1_3.4 target in the pCLS1107 expression vector using standard protocols and was used to transform *E. coli*. The resulting plasmid DNA was then used to transform yeast strains

expressing a variant cutting the HIV1_3.3 target in the pCLS0542 expression vector. Transformants were selected on synthetic medium lacking leucine and containing G418.

c) Mating of meganucleases coexpressing clones and screening in yeast

5 Mating was performed using a colony gridder (QpixII, Genetix). Variants were gridded on nylon filters covering YPD plates, using a low gridding density (4-6 spots/cm²). A second gridding process was performed on the same filters to spot a second layer consisting of different reporter-harboring yeast strains for each target. Membranes were placed on solid agar YPD rich medium, and incubated at
10 30°C for one night, to allow mating. Next, filters were transferred to synthetic medium, lacking leucine and tryptophan, adding G418, with galactose (2 %) as a carbon source, and incubated for five days at 37 °C, to select for diploids carrying the expression and target vectors. After 5 days, filters were placed on solid agarose medium with 0.02 % X-Gal in 0.5 M sodium phosphate buffer, pH 7.0, 0.1 % SDS,
15 6% dimethyl formamide (DMF), 7mM β-mercaptoethanol, 1% agarose, and incubated at 37°C, to monitor β-galactosidase activity. Results were analyzed by scanning and quantification was performed using appropriate software.

B) Results

Co-expression of variants cleaving the HIV1_3.4 target (4 variants)
20 and five variants cleaving the HIV1_3.3 target didn't result in cleavage of the HIV1_3 target, though most of the couples were able to cleave the HIV1_3.2 target.

Example 12: Improvement of meganucleases cleaving HIV1_3.3 by random mutagenesis of initial proteins cleaving HIV1_3.3

I-CreI variants able to cleave the HIV1_3.3 target have been
25 previously identified in example 9.

These variants display, however, weak cleavage activity and were therefore mutagenized in order to improve their activity. Four mutants were selected for random mutagenesis and the variants obtained were screened for cleavage activity of HIV1_3.3 and HIV1_3.5 targets. According to the structure of the I-CreI protein
30 bound to its target, there is no contact between the 4 central base pairs (positions -2 to 2) and the I-CreI protein (Chevalier *et al.*, Nat. Struct. Biol., 2001, 8, 312-316; Chevalier and Stoddard, Nucleic Acids Res., 2001, 29, 3757-3774; Chevalier *et al.*, J.

Mol. Biol., 2003, 329, 253-269). Thus, it is difficult to rationally choose a set of positions to mutagenize, and mutagenesis was performed on the whole protein.

A) Material and Methods

a) Construction of libraries by random mutagenesis

5 Random mutagenesis was performed on a pool of chosen variants, by PCR using Mn^{2+} . PCR reactions were carried out that amplify the I-CreI coding sequence using the primers preATGCreFor (5'-gcataaattactatacttctatagacacgcaaacacaaatacacagcgcccttgccacc-3'; SEQ ID NO: 24) and ICreIpostRev (5'-ggctcgaggagctcgtctagaggatcgctcgagttatcagtcggccgc-3'; SEQ ID NO: 25), which are common to the pCLS0542 (Figure 9) and pCLS1107 (Figure 11) vectors. Approximately 25 ng of the PCR product and 75 ng of vector DNA (pCLS0542) linearized by digestion with *NcoI* and *EagI* were used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (*MAT α* , *trp1* Δ 63, *leu2* Δ 1, *his3* Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, 10 Methods Enzymol., 2002, 350, 87-96). Expression plasmids containing an intact coding sequence for the I-CreI variant were generated by *in vivo* homologous recombination in yeast.

b) Mating of meganuclease expressing clones and screening in yeast

20 Experiments were performed as previously described in example 9. Positive resulting clones were verified by sequencing (MILLEGEN) as described in example 9.

B) Results

25 Four variants cleaving HIV1_3.3, were pooled, randomly mutagenized and transformed into yeast. The sequences of the variants subjected to random mutagenesis are described in table XVI (SEQ ID 88 to 91).

2232 transformed clones were screened for cleavage against the HIV1_3.3 and HIV1_3.5 DNA targets. A total of 51 positive clones were found to cleave HIV1_3.3, while none of those cleaved the HIV1_3.5 target. Sequencing of the 51 clones allowed the identification of 35 novel endonuclease variants. An example of 30 the identified variants is presented in table XIX and in figure 27.

Table XIX: Examples of 10 functional variants displaying strong cleavage activity for HIV1_3.3 after random mutagenesis.

Optimized variants HIV1_3.3	
SEQ ID NO: 105	32K 33A 44K 50R 68E 70S 75N 77R
SEQ ID NO: 106	32K 33A 44K 54I 60G 68E 70S 75N 77R 83T
SEQ ID NO: 107	32K 33A 44K 54L 68E 70S 75N 77R
SEQ ID NO: 108	32K 33A 44K 68E 70S 75N 77R 96R 105A 150S
SEQ ID NO: 109	32K 33A 44K 68E 70S 75N 77R 132N
SEQ ID NO: 110	33S 38Y 40R 44K 68S 70N 102V
SEQ ID NO: 111	33S 38Y 40R 44K 68S 70N
SEQ ID NO: 112	24V 32K 33A 35Y 44K 68E 70S 75N 77R
SEQ ID NO: 113	32K 33A 44K 68E 70S 75N 77R 81V 85R 154C
SEQ ID NO: 114	30C 33C 44K 54L 68A 70S 77K

* Mutations resulting from random mutagenesis are in bold.

5

Example 12bis: Improvement of meganucleases cleaving HIV1_3.3 by a second round of random mutagenesis of proteins cleaving HIV1_3.3

In order to further improve the activity of the obtained meganucleases, a second round of random mutagenesis was carried out following the same rationale of example 7bis. For this purpose, ten variants cleaving HIV1_3.3 were mutagenized, and variants were screened for cleavage activity of HIV1_3.3 and HIV1_3.5 targets. The materials and methods have previously been described in example 11.

A) Results

Ten variants cleaving HIV1_3.3, were pooled, randomly mutagenized and transformed into yeast. The variants submitted to random mutagenesis correspond to variants described in Table XIX (SEQ ID NO: 105 to 114).

2232 transformed clones were screened for cleavage against the HIV1_3.3 and HIV1_3.5 DNA targets. A total of 262 positive clones were found to cleave HIV1_3.3, while 24 of those cleaved also, though weakly, the HIV1_3.5 target. Sequencing of the 93 clones showing the strongest cleavage activity in the HIV1_3.3 target allowed the identification of 69 novel endonuclease variants. An example of the identified variants is presented in table XX and figure 28.

Table XX: Examples of 10 functional variants displaying strong cleavage activity for HIV1_3.3.

Optimized variants HIV1_3.3 (2nd round)	
SEQ ID NO: 115	32K 33A 44K 68E 70S 75N 77R 96R 105A 154R
SEQ ID NO: 116	32K 33A 44K 68E 70S 72T 75N 77R 81V 85R 154C
SEQ ID NO: 117	32K 33A 43L 44K 54L 68E 70S 75N 77R
SEQ ID NO: 118	32K 33A 44K 49A 68E 70S 75N 77R 81V 85R 89A 129A 154C 158Q
SEQ ID NO: 119	30C 33C 44K 54L 68E 70S 75N 77R
SEQ ID NO: 120	16L 32K 33A 43L 44K 50R 68E 70S 75N 77R 81V 154C 155P
SEQ ID NO: 121	24V 32K 33A 44K 68E 70S 75N 77R 81V 85R 87L 154C 158E
SEQ ID NO: 122	7E 33S 38Y 40R 44K 68E 70S 75Y 77R 96R 105A
SEQ ID NO: 123	32K 33A 44K 50R 68E 70S 75N 77R
SEQ ID NO: 124	2S 32K 33A 44K 68E 70S 75N 77R 132N

* Mutations resulting from random mutagenesis are in bold.

5

Example 13: Improvement of meganucleases cleaving HIV1_3 by site-directed mutagenesis of proteins cleaving HIV1_3.3 and assembly with proteins cleaving HIV1_3.4

Five I-CreI variants cleaving HIV1_3.3 after two cycles of random mutagenesis (examples 12 and 12bis) were mutagenized by introducing selected amino-acid substitutions in the proteins and screening for more efficient variants cleaving HIV1_3 in combination with a variant cleaving HIV1_3.4.

Six amino-acid substitutions have been found in previous studies to enhance the activity of I-CreI derivatives: these mutations correspond to the replacement of Glycine 19 with Serine (G19S), Phenylalanine 54 with Leucine (F54L), Glutamic acid 80 with Lysine (E80K), Phenylalanine 87 with Leucine (F87L), Valine 105 with Alanine (V105A) and Isoleucine 132 with Valine (I132V). These mutations were introduced into the coding sequence of proteins cleaving HIV1_3.3, and the resulting proteins were tested for their ability to induce cleavage of the HIV1_3 target, upon co-expression with a variant cleaving HIV1_3.4, as well as for the ability to cleave targets HIV1_3.3 and HIV1_3.5.

A) Material and Methods

a) Site-directed mutagenesis

Site-directed mutagenesis libraries were created by PCR on a pool of chosen variants. For example, to introduce the G19S substitution into the coding sequence of the variants, two separate overlapping PCR reactions were carried

25

out that amplify the 5' end (residues 1-24) or the 3' end (residues 14-167) of the I-*CreI* coding sequence. For both the 5' and 3' end, PCR amplification is carried out using a primer with homology to the vector (Gal10F 5'-gcaactttagtgctgacacatacagg-3' (SEQ ID NO: 16) or Gal10R 5'-acaaccttgattggagactgacc-3' (SEQ ID NO: 17) and a
 5 primer specific to the I-*CreI* coding sequence for amino acids 14-24 that contains the substitution mutation G19S (G19SF 5'-gccggcttggactctgacggtagcatcatc-3' (SEQ ID NO: 47) or G19SR 5'-gatgatgctaccgtcagagtccacaagccggc-3' (SEQ ID NO: 48)).

The same strategy is used with the following pair of oligonucleotides to introduce the mutations leading to the F54L, E80K, F87L, V105A and I132V substitutions in the
 10 coding sequences of the variants, respectively:

* F54LF: 5'-accagcgccgttggtgctggacaaactagtg-3' and F54LR: 5'-cactagttgtccagcagccaacggcgctgggt-3' (SEQ ID NO: 49 and 50);

* E80KF: 5'-ttaagcaaaatcaagccgtgcacaacttctg-3' and E80KR: 5'-caggaagttgtgcagcggctgattttgcttaa-3' SEQ ID NO: 51 and 52);

15 * F87LF: 5'-aagccgctgcacaacctgctgactcaactgcag-3' and F87LR: 5'-ctgcagttgagtcagcaggttgctgcagcggctt-3' SEQ ID NO: 53 and 54);

* V105AF: 5'-aaacaggcaaacctggtctgaaaattatcgaa-3' and V105AR: 5'-ttcgataatttcagagccaggtttgcctgtt-3' SEQ ID NO: 55 and 56);

* I132VF: 5'-acctgggtggatcaggttgagctctgaacgat-3' and I132VR: 5'-atcggtcagagctgcaacctgatccaccaggt-3' SEQ ID NO: 57 and 58).

For each substitution to be introduced, the resulting PCR products contain 33bp of homology with each other. The PCR fragments were purified. The ten PCR fragments were pooled in equimolar amounts to generate a mix containing 50ng of PCR DNA and 75ng of vector DNA (pCLS0542, Figure 9), linearized by digestion
 25 with *NcoI* and *EagI*. This mix was used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (*MAT α* , *trp1*Δ63, *leu2*Δ1, *his3*Δ200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). Intact coding sequences containing the substitutions are generated *in vivo* by homologous recombination in yeast.

30 c) Mating of meganuclease expressing clones and screening in yeast

The experimental procedure is as described in example 11.

d) Sequencing of variants

The experimental procedure is as described in example 9.

B) Results

A library containing a population harboring the six amino-acid
 5 substitutions (Glycine 19 with Serine, Phenylalanine 54 with Leucine, Glutamic acid
 80 with Lysine, Phenylalanine 87 with Leucine, Valine 105 with Alanine and
 Isoleucine 132 with Valine) was constructed on a pool of five variants cleaving
 HIV1_3.3 (described in Table XX, SEQ ID 115 to 119). 558 transformed clones were
 screened for cleavage against the HIV1_3.3 and HIV1_3.5 DNA targets. A total of
 10 376 positive clones were found to cleave HIV1_3.3, while 54 of those cleaved also the
 HIV1_3.5 target. An example of positive variants is shown in figure 29.

The 558 transformed clones were also mated with a yeast strain that
 contains (i) the HIV1_3 target in a reporter plasmid (ii) an expression plasmid
 containing a variant that cleaves the HIV1_3.4 target
 15 (38Y,44Y,68S,70S,75R,77V,43L,81V,105A,107R or KNSYYYS/YSSRV
 +43L+81V+105A+107R, according to the nomenclature of Table I). After mating
 with this yeast strain, 386 clones were found to cleave the HIV1_3. Thus, 386
 positives contained proteins able to form heterodimers with KNSYYYS/YSSRV
 +43L+81V+105A+107R (SEQ ID NO: 125) showing cleavage activity on the HIV1_3
 20 target. An example of positives is shown in Figure 30.

Sequencing of 93 clones with the high cleavage activity on the
 HIV1_3 and/or HIV1_3.3 target allowed the identification of 62 different
 endonuclease variants.

As an example, ten I-CreI variants cleaving the HIV1_3 target when
 25 forming a heterodimer with the KNSYYYS/YSSRV variant are listed in Table XXI.

Table XXI: Functional variant combinations displaying strong cleavage activity for HIV1_3.

		Optimized* Variants HIV1_3.3 (SEQ ID NO: 126 to 135, in this order)
VARIANT HIV1_3.4 (SEQ ID NO:125)	I-CreI 38Y,44Y,68S,70S,75R,77V,43L,81V,105A, 107R KNSTQK/RYSND +43L+81V+105A+107R	32K 33A 44K 68E 70S 75N 77R 80K 154R
		32K 33A 44K 68E 70S 72T 75N 77R 80K 129A 154C 158Q
		6D 19S 32K 33A 44K 49A 68E 70S 75N 77R 81V 85R 89A 129A 132V 154R
		32K 33A 44K 68E 70S 72T 75N 77R 80K 89A 105A
		19S 32K 33A 43L 44K 49A 68E 70S 75N 77R 81V 85R 89A 129A 154C 158Q
		32K 33A 44K 68E 70S 75N 77R 80K 96R 105A 132V 154C
		19S 32K 33A 44K 68E 70S 72T 73I 75N 77R 81V 85R 105A
		19S 30C 33C 44K 54L 68E 70S 75N 77R
		32K 33A 43L 44K 54L 68E 70S 75N 77R 80K 154C
		19S 32K 33A 44K 68E 70S 72T 75N 77R 80K 92R 96R 105A 154R

* Mutations resulting from site-directed mutagenesis are in bold.

Example 14: Improvement of meganucleases cleaving HIV1_3.4 by random mutagenesis of initial proteins cleaving HIV1_3.4

As a complement to example 5 we also decided to perform random mutagenesis with variants that cleave HIV1_3.4. The mutagenized proteins cleaving HIV1_3.4 were then tested to determine the efficiency of cleavage of the HIV1_3.4 and HIV1_3.6 targets.

A) Material and Methods

a) Construction of libraries by random mutagenesis

Random mutagenesis was performed on a pool of chosen variants, by PCR using Mn^{2+} . PCR reactions were carried out that amplify the I-CreI coding sequence using the primers preATGCreFor (5'-gcataaattactatacttctatagacacgcaaacacaaatacacagcggccttgccacc-3'; SEQ ID NO: 24) and ICReIpostRev (5'-ggctcgaggagctcgcttagaggatcgctcgagttatcagtcggccgc-3'; SEQ ID NO: 25). Approximately 25 ng of the PCR product and 75 ng of vector DNA (pCLS1107, Figure 11) linearized by digestion with *Dra*III and *Ngo*MIV were used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (*MAT α* , *trp1* Δ 63, *leu2* Δ 1, *his3* Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). Expression plasmids containing an intact coding sequence for the I-CreI variant were generated by *in vivo* homologous recombination in yeast.

b) Mating of meganuclease expressing clones and screening in yeast

Mating was performed as previously described in example 9. Positive resulting clones were verified by sequencing (MILLEGEN) as described in example 9.

5 **B) Results**

Five variants cleaving HIV1_3.4 were pooled, randomly mutagenized and transformed into yeast. The sequences of the variants subjected to random mutagenesis are described in table XVIII (SEQ ID NO:97 to 101).

2232 transformed clones were screened for cleavage against the HIV1_3.4 and HIV1_3.6 DNA targets. A total of 645 positive clones were found to cleave HIV1_3.4, while 156 of those also cleaved the HIV1_3.6 target. Sequencing of the 93 clones showing the strongest activity allowed the identification of 52 novel endonuclease variants. An example of the identified variants is presented in table XXII and in figure 31

15 **Table XXII: Examples of 10 functional variants displaying strong cleavage activity for HIV1_3.4.**

Optimized variants HIV1_3.4	
SEQ ID NO: 136	38Y 43L 44Y 68S 70S 75R 77V 81V 105A 107R
SEQ ID NO: 137	38Y 44Y 68S 70S 75R 77V 105A 132V
SEQ ID NO: 138	38Y 43L 44Y 68S 70S 75R 77V 105A
SEQ ID NO: 139	30R 38E 40R 44N 70S 75R 77N 94Y 105A
SEQ ID NO: 140	30R 38E 40R 44N 70S 75R 77N 105A
SEQ ID NO: 141	38Y 44Y 54I 68S 70S 75R 77V 105A
SEQ ID NO: 142	38Y 44Y 68S 70S 75R 77V 80G 105A
SEQ ID NO: 143	30R 38E 40R 44N 66C 70S 75R 77N
SEQ ID NO: 144	38Y 44Y 46S 68S 70S 75R 77V 96R 105A
SEQ ID NO: 145	30R 38E 40R 44N 70S 75R 77N 105A

* Mutations resulting from random mutagenesis are in bold.

20 **Example 14bis: Improvement of meganucleases cleaving HIV1_3.4 by a second round of random mutagenesis of proteins cleaving HIV1_3.3**

In order to further improve the activity of the obtained meganucleases, a second round of random mutagenesis was carried out following the same rationale of example 6. For this purpose, ten variants cleaving HIV1_3.4 were mutagenized, and variants were screened for cleavage activity of HIV1_3.4 and HIV1_3.6 targets. The materials and methods have previously been described in example 11.

A) Results

Ten variants cleaving HIV1_3.4, were pooled, randomly mutagenized and transformed into yeast. The variants submitted to random mutagenesis correspond to variants described in Table XXII (SEQ ID NO: 136 to 145).

2232 transformed clones were screened for cleavage against the HIV1_3.4 and HIV1_3.6 targets. A total of 178 positive clones were found to cleave HIV1_3.4, while 63 of those cleaved also the HIV1_3.6 target. Sequencing of the 93 clones showing the strongest cleavage activity in the HIV1_3.4 target allowed the identification of 62 novel endonuclease variants. An example of the identified variants is presented in table XXIII and figure 32.

Table XXIII: Examples of 10 functional variants displaying strong cleavage activity for HIV1_3.4.

Optimized variants HIV1_3.4 (2nd round)	
SEQ ID NO: 146	30R 38E 40R 44N 64G 70S 75R 77N 105A 114T 153G
SEQ ID NO: 147	24V 38Y 43L 44Y 68S 70S 75R 77V 105A 153G 160R
SEQ ID NO: 148	4I 38Y 44Y 54I 68S 70S 75R 77V 105A
SEQ ID NO: 149	38Y 40C 44Y 46S 68S 70S 75R 77V 81V 92R 96R 105A
SEQ ID NO: 150	33C 38S 70S 75N 77K
SEQ ID NO: 151	30R 34R 38E 40R 44N 70S 75R 77N 94Y 105A
SEQ ID NO: 152	38Y 44Y 54I 68S 70S 75R 77V 105A 162F
SEQ ID NO: 153	7R 38Y 40C 44Y 54I 68S 69V 70S 75R 77V 105A
SEQ ID NO: 154	38Y 44Y 54I 68S 70S 75R 77V 105A 120G 160R
SEQ ID NO: 155	30R 38E 40R 44N 70S 75R 77N 105A 157K

* Mutations resulting from random mutagenesis are in bold.

Example 15: Improvement of meganucleases cleaving HIV1_3 by site-directed mutagenesis of proteins cleaving HIV1_3.4 and assembly with proteins cleaving HIV1_3.3

Four of the improved I-CreI variants cleaving HIV1_3.4 described in Table XXIII and used for a second round of random mutagenesis in example 14bis were also mutagenized by introducing selected amino-acid substitutions in the proteins and screening for variants cleaving HIV1_3.1 in combination with a variant cleaving HIV1_3.3.

Six amino-acid substitutions have been found in previous studies to enhance the activity of I-CreI derivatives: these mutations correspond to the replacement of Glycine 19 with Serine (G19S), Phenylalanine 54 with Leucine (F54L), Glutamic acid 80 with Lysine (E80K), Phenylalanine 87 with Leucine (F87L), Valine 105 with Alanine (V105A) and Isoleucine 132 with Valine (I132V). These mutations

were introduced into the coding sequence of proteins cleaving HIV1_3.3, and the resulting proteins were tested for their ability to induce cleavage of the HIV1_3 target, upon co-expression with a variant cleaving HIV1_3.4.

A) Material and Methods

- 5 Site-directed mutagenesis libraries were created by PCR on a pool of chosen variants. For example, to introduce the G19S substitution into the coding sequence of the variants, two separate overlapping PCR reactions were carried out that amplify the 5' end (residues 1-24) or the 3' end (residues 14-167) of the I-CreI coding sequence. For both the 5' and 3' end, PCR amplification is carried out using a primer
- 10 with homology to the vector (Gal10F 5'-gcaactttagtgctgacacatacagg-3' (SEQ ID NO: 16) or Gal10R 5'-acaaccttgattggagacttgacc-3' (SEQ ID NO: 17) and a primer specific to the I-CreI coding sequence for amino acids 14-24 that contains the substitution mutation G19S (G19SF 5'-gccggcttggactctgacggtagcatcatc-3' (SEQ ID NO: 47) or G19SR 5'-gatgatgctaccgtcagagtccacaaagccggc-3' (SEQ ID NO: 48)).
- 15 The same strategy is used with the following pair of oligonucleotides to introduce the mutations leading to the F54L, E80K, F87L, V105A and I132V substitutions in the coding sequences of the variants, respectively:
- * F54LF: 5'-accagcgccgttggctgctggacaaactagt-3' and F54LR: 5'-cactagttgtccagcagccaacggcgctgggt-3' (SEQ ID NO: 49 and 50);
- 20 * E80KF: 5'-ttaagcaaaatcaagccgctgcacaacttctg-3' and E80KR: 5'-caggaagttgtgcagcggttgatttgcctaa-3' SEQ ID NO: 51 and 52);
- * F87LF: 5'-aagccgctgcacaacctgctgactcaactgcag-3' and F87LR: 5'-ctgcagttgagtcagcaggttgcagcggtt-3' SEQ ID NO: 53 and 54);
- * V105AF: 5'-aaacaggcaaacctggctctgaaaattatcgaa-3' and V105AR: 5'-ttcgataatttcagagccaggttgcctgtt-3' SEQ ID NO: 55 and 56);
- 25 * I132VF: 5'-acctgggtggatcaggttgcagctctgaacgat-3' and I132VR: 5'-atcggtcagagctgcaacctgatccaccaggt-3' SEQ ID NO: 57 and 58).

For each substitution to be introduced, the resulting PCR products contain 33bp of homology with each other. The PCR fragments were purified. The ten

30 PCR fragments were pooled in equimolar amounts to generate a mix containing 50ng of PCR DNA and 75ng of vector DNA (pCLS0542, Figure 9), linearized by digestion with *NcoI* and *EagI*. This mix was used to transform the yeast *Saccharomyces*

cerevisiae strain FYC2-6A (*MAT α* , *trp1* Δ 63, *leu2* Δ 1, *his3* Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). Intact coding sequences containing the substitutions are generated *in vivo* by homologous recombination in yeast.

5 c) Mating of meganuclease expressing clones and screening in yeast

The experimental procedure is as described in example 11.

d) Sequencing of variants

The experimental procedure is as described in example 9.

B) Results

10 A library containing a population harboring the six amino-acid substitutions (Glycine 19 with Serine, Phenylalanine 54 with Leucine, Glutamic acid 80 with Lysine, Phenylalanine 87 with Leucine, Valine 105 with Alanine and Isoleucine 132 with Valine) was constructed on a pool of four variants cleaving HIV1_3.4 (SEQ ID 136 to 139, Table XXII). 317 transformed clones were screened
15 for cleavage against the HIV1_3.4 and HIV1_3.6 DNA targets. A total of 311 positive clones were found to cleave HIV1_3.4, while 262 of those cleaved also the HIV1_3.6 target. An example of positive variants is shown in figure 33.

The 317 transformed clones were also mated with a yeast strain that contains (i) the HIV1_3 target in a reporter plasmid (ii) an expression plasmid
20 containing a variant that cleaves the HIV1_3.3 target (I-CreI 32K,33A,44K,68E,70S,75N,77R, +132N or KNKAQS/KESNR +132N, according to the nomenclature of Table I). After mating with this yeast strain, 264 clones were found to cleave the HIV1_3. Thus, 264 positives contained proteins able to form heterodimers with KNKAQS/KESNR +132N (SEQ ID NO: 109, Table XIX) showing
25 cleavage activity on the HIV1_3 target. An example of positive clones is shown in Figure 34.

Sequencing of the 317 clones allowed the identification of 69 different endonuclease variants.

As an example, ten I-CreI variants cleaving the HIV1_3 target when
30 forming a heterodimer with the KNKAQS/KESNR +132N variant are listed in Table XXIV.

Table XXIV: Functional variant combinations displaying cleavage activity for HIV1_3 target

		Optimized* Variants HIV1_3.4 (SEQ ID NO: 156 to 165)
VARIANT HIV1_3.3	I-CreI 28K30N32K33A38Q40S 44K68E70S75N77R +132N (KNKAQS/KESNR +132N) (SEQ ID NO: 109)	38Y 44Y 68S 70S 75R 77V 80K 105A
		28S 40K 43L 44L 70N 75N 80K 132V
		38Y 43L 44Y 68S 70S 75R 77V 80K 105A 132V
		38Y 43L 44Y 68S 70S 75R 77V 80K 94Y 105A
		38Y 43L 44Y 68S 70S 75R 77V 80K 105A
		30R 38E 40R 44Y 68S 70S 75R 77V 105A 132V
		38Y 43L 44Y 68S 70S 75R 77V 80K 105A 107R 132V
		19S 38Y 43L 44Y 68S 70S 75R 77V 105A 132V
		38Y 43L 44Y 68S 70S 75R 77V 80K 105A 132V
		38Y 43L 44Y 68S 70S 75R 77V 105A 132V

* Mutations resulting from site-directed mutagenesis are in bold.

5 **Example 16: Strategy for engineering meganucleases cleaving the HIV1_4 target from the HIV1 virus**

The HIV1_4 target is a 22 bp (non-palindromic) target located in the gag gene of the HIV1 provirus. This target is precisely located at positions 1629-1650 of the HIV-1 pNL4-3 vector (accession number AF324493, Adachi et al., J. Virol., 1986, 59, 284-291), a subtype B infectious molecular clone.

10 The HIV1_4 sequence (SEQ ID NO: 331) is partly a patchwork of the 10AGC_P, 10TGT_P, 5TCT_P and 5_TAT_P targets (Figure 35) which are cleaved by previously identified meganucleases, obtained as described in International PCT Applications WO 2006/097784 and WO 2006/097853; Arnould et al., J. Mol. Biol., 2006, 355, 443-458; Smith et al., Nucleic Acids Res., 2006. Thus, HIV1_4 could be
15 cleaved by combinatorial variants resulting from these previously identified meganucleases.

20 The 10AGC_P, 10TGT_P, 5TCT_P and 5_TAT_P target sequences are 24 bp derivatives of C1221, a palindromic sequence cleaved by I-CreI (Arnould et al., precited). However, the structure of I-CreI bound to its DNA target suggests that the two external base pairs of these targets (positions -12 and 12) have no impact on binding and cleavage (Chevalier et al., Nat. Struct. Biol., 2001, 8, 312-316; Chevalier and Stoddard, Nucleic Acids Res., 2001, 29, 3757-3774; Chevalier et al., J. Mol. Biol.,

2003, 329, 253-269), and in this study, only positions -11 to 11 were considered. Consequently, the HIV1_4 series of targets were defined as 22 bp sequences instead of 24 bp. HIV1_4 differs from C1221 in the 4 bp central region. According to the structure of the I-CreI protein bound to its target, there is no contact between the 4 central base pairs (positions -2 to 2) and the I-CreI protein (Chevalier et al., Nat. Struct. Biol., 2001, 8, 312-316; Chevalier and Stoddard, Nucleic Acids Res., 2001, 29, 3757-3774; Chevalier et al., J. Mol. Biol., 2003, 329, 253-269). Thus, the bases at these positions should not impact the binding efficiency. However, they could affect cleavage, which results from two nicks at the edge of this region. Thus, the GGAC sequence in -2 to 2 was first substituted with the GTAC sequence from C1221, resulting in target HIV1_4.2 (SEQ ID NO: 332, Figure 35). Then, two palindromic targets, HIV1_4.3 (SEQ ID NO: 333) and HIV1_4.4 (SEQ ID NO: 334), were derived from HIV1_4.2 (Figure 35). Since HIV1_4.3 and HIV1_4.4 are palindromic, they should be cleaved by homodimeric proteins. Two other pseudo-palindromic targets were derived from these two, containing the GGAC sequence in -2 to 2 (targets HIV1_4.5 (SEQ ID NO: 335) and HIV1_4.6 (SEQ ID NO: 336), figure 35). Thus, proteins able to cleave HIV1_4.3 and HIV1_4.4 targets or, preferentially, the pseudo-palindromic targets as homodimers were first designed (examples 17 and 18) and then co-expressed to obtain heterodimers cleaving HIV1_4 (example 19). Heterodimers cleaving the HIV1_4.2 and HIV1_4 targets could be identified. In order to improve cleavage activity for the HIV1_4 target, a series of variants cleaving HIV1_4.3 and HIV1_4.4 was chosen, and then refined. The chosen variants were subjected to random or site-directed mutagenesis, and used to form novel heterodimers that were screened against the HIV1_4 target (examples 20, 21, 22 and 23). Heterodimers could be identified with an improved cleavage activity for the HIV1_4 target.

Example 17: Identification of meganucleases cleaving HIV1_4.3

This example shows that I-CreI variants can cut the HIV1_4.3 DNA target sequence derived from the left part of the HIV1_4.2 target in a palindromic form (Figure 35).

HIV1_4.3 is similar to 10AGC_P at positions ± 1 , ± 2 , ± 6 , ± 8 , ± 9 , and ± 10 and to 5TCT_P at positions ± 1 , ± 2 , ± 3 , ± 4 , ± 5 and ± 6 . It was hypothesized that positions ± 7 and ± 11 would have little effect on the binding and cleavage activity.

Variants able to cleave the 10AGC_P target were obtained by mutagenesis of I-CreI N75 or D75, at positions 28, 30, 32, 33, 38, 40 and 70, as described previously in Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156. Variants able to cleave 5TCT_P were obtained
 5 by mutagenesis on I-CreI N75 at positions 24, 44, 68, 70, 75 and 77 as described in Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2006/097784, WO 2006/097853, WO 2007/060495 and WO 2007/049156.

Both sets of proteins are mutated at position 70. However, the
 10 existence of two separable functional subdomains was hypothesized. This implies that this position has little impact on the specificity at bases 10 to 8 of the target. Mutations at positions 24 found in variants cleaving the 5TCT_P target will be lost during the combinatorial process. But it was hypothesized that this will have little impact on the capacity of the combined variants to cleave the HIV1_4.3 target.

15 Therefore, to check whether combined variants could cleave the HIV1_4.3 target, mutations at positions 44, 68, 70, 75 and 77 from proteins cleaving 5TCT_P were combined with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10AGC_P.

A) Material and Methods

20 a) Construction of target vector

The target was cloned as follows: an oligonucleotide corresponding to the HIV1_4.3 target sequence flanked by gateway cloning sequences was ordered from PROLIGO: 5' TGGCATACAAGTTTCCAGCATTCTGTACAGAATGCTGGCAATCGTCTGTCA 3' (SEQ ID NO: 166). The same procedure was followed for cloning the HIV1_4.5
 25 target, using the oligonucleotide: 5'TGGCATACAAGTTTCCAGCATTCTGGACAGAATGCTGGCAATCGTCTGTCA 3' (SEQ ID NO: 167). Double-stranded target DNA, generated by PCR amplification of the single stranded oligonucleotide, was cloned using the Gateway protocol (INVITROGEN) into the yeast reporter vector (pCLS1055, Figure 8). Yeast reporter
 30 vector was transformed into *Saccharomyces cerevisiae* strain FYBL2-7B (*MAT a*, *ura3*Δ851, *trp1*Δ63, *leu2*Δ1, *lys2*Δ202), resulting in a reporter strain.

b) Construction of combinatorial mutants

I-CreI variants cleaving 10AGC_P or 5TCT_P were previously identified, as described in Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156, and Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; International PCT Applications WO 2006/097784 and WO 2006/097853, respectively for the 10AGC_P and 5TCT_P targets. In order to generate I-CreI derived coding sequences containing mutations from both series, separate overlapping PCR reactions were carried out that amplify the 5' end (aa positions 1-43) or the 3' end (positions 39-167) of the I-CreI coding sequence. For both the 5' and 3' end, PCR amplification is carried out using primers (Gal10F 5'-gcaacttagtgctgacacatacagg-3' (SEQ ID NO: 16) or Gal10R 5'-acaaccttgattggagacttgacc-3'(SEQ ID NO: 17)) specific to the vector (pCLS0542, Figure 9) and primers (assF 5'-ctannnttgacctt-3' (SEQ ID NO: 18) or assR 5'-aaaggtcaannntag-3'(SEQ ID NO: 19)), where nnn codes for residue 40, specific to the I-CreI coding sequence for amino acids 39-43. The PCR fragments resulting from the amplification reaction realized with the same primers and with the same coding sequence for residue 40 were pooled. Then, each pool of PCR fragments resulting from the reaction with primers Gal10F and assR or assF and Gal10R was mixed in an equimolar ratio. Finally, approximately 25 ng of each final pool of the two overlapping PCR fragments and 75 ng of vector DNA (pCLS0542, Figure 9) linearized by digestion with *NcoI* and *EagI* were used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (MAT α , trp1 Δ 63, leu2 Δ 1, his3 Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). An intact coding sequence containing both groups of mutations is generated by *in vivo* homologous recombination in yeast.

c) Mating of meganuclease expressing clones and screening in yeast

Screening was performed as described previously (Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458). Mating was performed using a colony gridded (QpixII, GENETIX). Variants were gridded on nylon filters covering YPD plates, using a low gridding density (4-6 spots/cm²). A second gridding process was performed on the same filters to spot a second layer consisting of the reporter-harboring yeast strain. Membranes were placed on solid agar YPD rich medium, and

incubated at 30 °C for one night, to allow mating. Next, filters were transferred to synthetic medium, lacking leucine and tryptophan, with galactose (2 %) as a carbon source, and incubated for five days at 37 °C, to select for diploids carrying the expression and target vectors. After 5 days, filters were placed on solid agarose medium with 0.02 % X-Gal in 0.5 M sodium phosphate buffer, pH 7.0, 0.1 % SDS, 6% dimethyl formamide (DMF), 7 mM β -mercaptoethanol, 1% agarose, and incubated at 37°C, to monitor β -galactosidase activity. Results were analyzed by scanning and quantification was performed using appropriate software.

d) Sequencing of variants

To recover the variant expression plasmids, yeast DNA was extracted using standard protocols and used to transform *E. coli*. Sequencing of variant ORFs was then performed on the plasmids by MILLEGEN SA. Alternatively, ORFs were amplified from yeast DNA by PCR (Akada et al., Biotechniques, 2000, 28, 668-670), and sequencing was performed directly on the PCR product by MILLEGEN SA.

B) Results

I-*CreI* combinatorial variants were constructed by associating mutations at positions 44, 68, 70, 75 and 77 from proteins cleaving 5TCT_P with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10AGC_P on the I-*CreI* scaffold, resulting in a library of complexity 3800. Examples of combinatorial variants are displayed in Table XXV. This library was transformed into yeast and 3348 clones were screened for cleavage against the HIV1_4.3 and HIV1_4.5 DNA targets. 7 positive clones were found to cleave the HIV1_4.3 target, which after sequencing turned out to correspond to 7 different novel endonuclease variants (Table XXVI). Those variants showed no cleavage activity of the HIV1_4.5 DNA target. Examples of positives are shown in Figure 36. Two of the variants obtained display non parental combinations at positions 28, 30, 32, 33, 38, 40 or 44, 68, 70, 75, 77 (SEQ ID 168 and 174, Table XXVI). Such combinations likely result from PCR artifacts during the combinatorial process. Alternatively, the variants may be I-*CreI* combined variants resulting from micro-recombination between two original variants during *in vivo* homologous recombination in yeast.

Table XXV: Panel of variants* theoretically present in the combinatorial library

Amino acids at positions 44, 68, 70, 75 and 77 (ex : ARNNI stands for A44, R68, N70, N75 and I77)	Amino acids at positions 28, 30, 32, 33, 38 and 40 (ex: KHSSQS stands for K28, H30, S32, S33, Q38 and S40)										
	KDSRQS	KRSPQS	KTYYS	QNSYRK	KNSGQQ	KNSTGS	KRDYQS	KNSGGS	KNSRQR	KTSYQR	KNSTTS
KQSNR											
KYSNV											
KASNI				+							
KNSNI											
KYSNQ											
QGGNI											
KAANI				+							
KNNNI											
KTSNV											
KRSNV											
KGGNI				+							
KQSNR											
KYSNV											
KTGNI				+			+				
KRGNI											
KNANI											
QASNR											
QSSNR											
KKANI											
PCSYT											
KKANI											
QNSNR											
KSSNV											
KTTNI											

*Only 264 out of the 3800 combinations are displayed.

+ indicates that a functional combinatorial variant cleaving the HIV1_4.3 target was found among the identified positives.

Table XXVI: I-CreI variants capable of cleaving the HIV1_4.3 DNA target.

Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77 of the I-CreI variants (ex: KRSRES/TYSNI stands for K28, R30, S32, R33, E38, S40/ T44, Y68, S70, N75 and I77)	SEQ ID NO:
QNSYRK/KRDNI	168
KRDYQS/KTGNI	169
QNSYRK/KTGNI	170
QNSYRK/KASNI	171
QNSYRK/KAANI	172
QNSYRK/KGGNI	173
KRSYQS/QASNR	174

Example 18: Making of meganucleases cleaving HIV1_4.4

This example shows that I-CreI variants can cleave the HIV1_4.4 DNA target sequence derived from the right part of the HIV1_4.2 target in a palindromic form (Figure 35).

5 HIV1_4.4 is similar to 5TAT_P at positions ± 1 , ± 2 , ± 3 , ± 4 , ± 5 and ± 8 and to 10TGT_P at positions ± 1 , ± 2 , ± 3 , ± 4 , ± 8 , ± 9 and ± 10 . It was hypothesized that positions ± 6 , ± 7 and ± 11 would have little effect on the binding and cleavage activity. Variants able to cleave 5TAT_P were obtained by mutagenesis of I-CreI N75 at positions 44, 68, 70, 75 and 77, as described previously (Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2006/097784, WO 2006/097853, WO 2007/060495 and WO 2007/049156). Variants able to cleave the 10TGT_P target were obtained by mutagenesis of I-CreI N75 or D75, at positions 28, 30, 32, 33, 38, 40 and 70, as described previously in Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156.

Both sets of proteins are mutated at position 70. However, the existence of two separable functional subdomains was hypothesized. This implies that this position has little impact on the specificity at bases 10 to 8 of the target.

Therefore, to check whether combined variants could cleave the HIV1_4.4 target, mutations at positions 44, 68, 70, 75 and 77 from proteins cleaving 5TAT_P were combined with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10TGT_P.

A) Material and Methods**a) Construction of target vector**

25 The experimental procedure is as described in example 17, with the exception that different oligonucleotides corresponding to the HIV1_4.4 and HIV1_4.6 targets. The oligonucleotide used for the HIV1_4.4 target was: 5'TGGCATAACAAGTTTCTTGTCTTATGTACATAAGACAAGCAATCGTCTGTCA3' (SEQ ID NO: 175),

30 and

5'TGGCATAACAAGTTTCTTGTCTTATGGACATAAGACAAGCAATCGTCTGTCA3' (SEQ ID NO: 176) for HIV1_4.6 target.

b) Construction of combinatorial variants

I-CreI variants cleaving 10TGT_P or 5STAT_P were previously identified, as described in Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156, and Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; International PCT Applications WO 2006/097784 and WO 2006/097853, respectively for the 10TGT_P and 5STAT_P targets. In order to generate I-CreI derived coding sequences containing mutations from both series, separate overlapping PCR reactions were carried out that amplify the 5' end (aa positions 1-43) or the 3' end (positions 39-167) of the I-CreI coding sequence. For both the 5' and 3' end, PCR amplification is carried out using primers (Gal10F 5'-gcaacttagtgctgacacatacagg-3' (SEQ ID NO: 16) or Gal10R 5'-acaaccttgattggagacttgacc-3' (SEQ ID NO: 17)) specific to the vector (pCLS1107, Figure 11) and primers (assF 5'-ctannnttgaccttt-3' (SEQ ID NO: 18) or assR 5'-aaaggtcaannntag-3' (SEQ ID NO: 19), where nnn codes for residue 40, specific to the I-CreI coding sequence for amino acids 39-43. The PCR fragments resulting from the amplification reaction realized with the same primers and with the same coding sequence for residue 40 were pooled. Then, each pool of PCR fragments resulting from the reaction with primers Gal10F and assR or assF and Gal10R was mixed in an equimolar ratio. Finally, approximately 25 ng of each final pool of the two overlapping PCR fragments and 75 ng of vector DNA (pCLS1107, Figure 11) linearized by digestion with DraIII and NgoMIV were used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (MAT α , trp1 Δ 63, leu2 Δ 1, his3 Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). An intact coding sequence containing both groups of mutations is generated by *in vivo* homologous recombination in yeast.

c) Mating of meganuclease expressing clones and screening in yeast

Screening was performed as described previously (Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458). Mating was performed using a colony gridder (QpixII, GENETIX). Variants were gridded on nylon filters covering YPD plates, using a low gridding density (4-6 spots/cm²). A second gridding process was performed on the same filters to spot a second layer consisting of the reporter-harboring yeast strain. Membranes were placed on solid agar YPD rich medium, and

incubated at 30 °C for one night, to allow mating. Next, filters were transferred to synthetic medium, lacking tryptophan, adding G418, with galactose (2 %) as a carbon source, and incubated for five days at 37 °C, to select for diploids carrying the expression and target vectors. After 5 days, filters were placed on solid agarose medium with 0.02 % X-Gal in 0.5 M sodium phosphate buffer, pH 7.0, 0.1 % SDS, 6% dimethyl formamide (DMF), 7 mM β -mercaptoethanol, 1% agarose, and incubated at 37°C, to monitor β -galactosidase activity. Results were analyzed by scanning and quantification was performed using appropriate software. Positives resulting clones were verified by sequencing (MILLEGEN) as described in example 2.

10 B) Results

I-*CreI* combinatorial variants were constructed by associating mutations at positions 44, 68, 70, 75 and 77 from proteins cleaving 5TAT_P with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10TGT_P on the I-*CreI* scaffold, resulting in a library of complexity 1406. Examples of combinatorial variants are displayed in Table XXVII. This library was transformed into yeast and 3348 clones (2.3 times the diversity) were screened for cleavage against the HIV1_4.4 and HIV1_4.6 DNA targets. A total of 210 positive clones were found to cleave HIV1_4.4. 40 of these clones were also able to cleave the HIV1_4.6 DNA target. Sequencing of these 93 clones with the strongest activity allowed the identification of 45 novel endonuclease variants. Examples of positives are shown in Figure 37. The sequence of several of the variants identified display non parental combinations at positions 28, 30, 32, 33, 38, 40 or 44, 68, 70, 75, 77 as well as additional mutations (see examples in Table XXVIII, SEQ ID 178 and 184). Such variants likely result from PCR artifacts during the combinatorial process. Alternatively, the variants may be I-*CreI* combined variants resulting from micro-recombination between two original variants during *in vivo* homologous recombination in yeast.

Table XXVII: Panel of variants* theoretically present in the combinatorial library

Amino acids at positions 44, 68, 70, 75 and 77 (ex : ARNNI stands for A44, R68, N70, N75 and I77)	Amino acids at positions 28, 30,32, 33, 38 and 40 (ex: KHSSQS stands for K28, H30, S32, S33, Q38 and S40)										
	ANSSRK	NNSSRK	QNSSRK	KHSCQS	KHSMAS	KHSTQS	KNDCQS	KNRAQS	KNSSRK	KNATQS	KNSSRS
AYSYK				+	+		+	+		+	
ARSYT	+		+								
YRSYN											
YYSYR	+		+								
ATGNI			+								
AKQNI											
ANANI			+								+
ARSYT											
AYSNI		+									
ARSNV											
NRGNI											
AASYR											+
ARSDY											
ANSYR			+								
ARNNI											
NHSYN											+
ARSYV											
ARGNI											
NYSYR					+	+		+	+	+	+
NRENI											
ASSYK											
NRSNT											
YYSNQ			+								+
YRSYQ											

* Only 264 out of the 1406 combinations are displayed.

5 + indicates that a functional combinatorial variant cleaving the HIV1_4.4 target was found among the identified positives.

Table XXVIII: I-CreI variants capable of cleaving the HIV1_4.4 DNA target.

Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77 of the I-CreI variants (ex: KRSRES/TYSNI stands for K28, R30, S32, R33, E38, S40/ T44, Y68, S70, N75 and I77)	SEQ ID NO:
KHSMAS/NYSYR	177
KNGTQS/AYSYR	178
KHSMAS/AYSYK	179
KNATQS/NYSYR	180
KNRAQS/NYSYR	181
KNSTQA/NYSYR	182
KNSGCS/NYSYR	183
ANSSRK/NYSYK +59A	184
ANSSRK/ARSYT	185
KHSCQS/AYSYK	186

Example 19: Making of meganucleases cleaving HIV1_4.2 and HIV1_4

5 I-CreI variants able to cleave each of the palindromic HIV1_4.2 derived targets (HIV1_4.3 and HIV1_4.4) were identified in example 2 and example 3. Pairs of such variants (one cutting HIV1_4.3 and one cutting HIV1_4.4) were co-expressed in yeast. Upon co-expression, there should be three active molecular species, two homodimers, and one heterodimer. It was assayed whether the
10 heterodimers that should be formed, cut the HIV1_4.2 and the non palindromic HIV1_4 targets.

A) Materials and Methods**a) Construction of target vector**

The experimental procedure is as described in example 2, with the exception that an
15 oligonucleotide corresponding to the HIV1_4.2 target sequence: 5'TGGCATACAAGTTTCCAGCATTCTGTACATAAGACAAGCAATCGTCTGTC A
3'(SEQ ID NO: 187) or the HIV1_4 target sequence: 5' TGGCATACAAGTTTCCAGCATTCTGGACATAAGACAAGCAATCGTCTGTC
20 A3'

(SEQ ID NO: 188) was used.

b) Co-expression of variants

Yeast DNA was extracted from variants cleaving the HIV1_4.4 target in the pCLS1107 expression vector using standard protocols and was used to transform *E. coli*. The resulting plasmid DNA was then used to transform yeast strains
5 expressing a variant cutting the HIV1_4.3 target in the pCLS0542 expression vector. Transformants were selected on synthetic medium lacking leucine and containing G418.

c) Mating of meganucleases coexpressing clones and screening in yeast

Mating was performed using a colony gridder (QpixII, Genetix).
10 Variants were gridded on nylon filters covering YPD plates, using a low gridding density (4-6 spots/cm²). A second gridding process was performed on the same filters to spot a second layer consisting of different reporter-harboring yeast strains for each target. Membranes were placed on solid agar YPD rich medium, and incubated at 30°C for one night, to allow mating. Next, filters were transferred to synthetic
15 medium, lacking leucine and tryptophan, adding G418, with galactose (2 %) as a carbon source, and incubated for five days at 37 °C, to select for diploids carrying the expression and target vectors. After 5 days, filters were placed on solid agarose medium with 0.02 % X-Gal in 0.5 M sodium phosphate buffer, pH 7.0, 0.1 % SDS, 6% dimethyl formamide (DMF), 7mM β-mercaptoethanol, 1% agarose, and incubated
20 at 37°C, to monitor β-galactosidase activity. Results were analyzed by scanning and quantification was performed using appropriate software.

B) Results

Co-expression of variants cleaving the HIV1_4.4 target (10 variants corresponding to those described in Table XXVIII, SEQ ID 177 to 186) and six
25 variants cleaving the HIV1_4.3 target (Table XXVI, SEQ ID 168 and 170 to 174) resulted in cleavage of the HIV1_4.2 target in most of the cases (Figure 38). Nevertheless, none of these combinations was able to cut the HIV1_4 natural target that differs from the HIV1_4.2 sequence by 2 bp at positions 1 and 2 (Figure 35). Examples of functional combinations are summarized in Table XXIX.

Table XXIX: Cleavage of the HIV1_4.2 target by the heterodimeric variants.

HIV1_4.2 target		Sequence of the I-CreI variants cleaving the HIV1_4.3 target [§]	
		QNSYRK/KTGNI SEQ ID NO:170	QNSYRK/KASNI SEQ ID NO:171
Sequence of the I-CreI variants cleaving the HIV1_4.4 target [§]	KHSMAS/NYSYR SEQ ID NO:177	+	+
	KNGTQS/AYSYR SEQ ID NO:178	+	+
	KHSMAS/AYSYK SEQ ID NO:179	+	+
	KNATQS/NYSYR SEQ ID NO:180	+	+
	KNRAQS/NYSYR SEQ ID NO:181	+	+
	KNSTQA/NYSYR SEQ ID NO:182	+	+

§ Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77 (ex: KRSRES/TYSNI stands for K28, R30, S32, R33, E38, S40/ T44, Y68, S70, N75 and I77)

+ indicates a functional combination

5 Example 20: Improvement of meganucleases cleaving HIV1_4.3 by random mutagenesis of proteins and assembly with proteins cleaving HIV1_4.4

The assembly of I-CreI variants cleaving the palindromic HIV1_4.3 and HIV1_4.4 target to cleave the HIV1_4.2 and HIV1_4 have been previously identified in example 4. However, these variants display activity with the HIV1_4.2
10 target and not with the HIV1_4 target.

Therefore seven variants cleaving HIV1_4.3 were mutagenized, and variants were screened for cleavage activity of HIV1_4.3 and HIV1_4.5 targets. Additionally the mutants with the strongest activity were screened for cleavage activity of HIV1_4 when co-expressed with a variant cleaving HIV1_4.4. According
15 to the structure of the I-CreI protein bound to its target, there is no contact between the 4 central base pairs (positions -2 to 2) and the I-CreI protein (Chevalier *et al.*, Nat. Struct. Biol., 2001, 8, 312-316; Chevalier and Stoddard, Nucleic Acids Res., 2001, 29, 3757-3774; Chevalier *et al.*, J. Mol. Biol., 2003, 329, 253-269). Thus, it is difficult to rationally choose a set of positions to mutagenize, and mutagenesis was performed on
20 the whole protein. Random mutagenesis results in high complexity libraries. Therefore, to limit the complexity of the variant libraries to be tested, only one of the two components of the heterodimers cleaving HIV1_4 was mutagenized.

Thus, in a first step, proteins cleaving HIV1_4.3 were mutagenized and their homodimeric cleavage activity was determined, and in a second step, it was

assessed whether they could cleave HIV1_4 when co-expressed with a protein cleaving HIV1_4.4.

A) Material and Methods

a) Construction of libraries by random mutagenesis

5 Random mutagenesis was performed on a pool of chosen variants, by PCR using Mn^{2+} . PCR reactions were carried out that amplify the I-CreI coding sequence using the primers preATGCreFor (5'-gcataaattactatacttctatagacacgcaaacacaaatacacagcggccttgccacc-3'; SEQ ID NO: 24) and ICrelpostRev (5'-ggctcgaggagctcgtctagaggatcgctcgagttatcagtcggccgc-3'; SEQ ID
10 NO: 25), which are common to the pCLS0542 (Figure 9) and pCLS1107 (Figure 11) vectors. Approximately 25 ng of the PCR product and 75 ng of vector DNA (pCLS0542) linearized by digestion with *NcoI* and *EagI* were used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (*MAT α* , *trp1* Δ 63, *leu2* Δ 1, *his3* Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods,
15 Methods Enzymol., 2002, 350, 87-96). Expression plasmids containing an intact coding sequence for the I-CreI variant were generated by *in vivo* homologous recombination in yeast.

b) Mating of meganuclease expressing clones and screening in yeast

Experiments were performed as previously described in
20 example 17. Positive resulting clones were verified by sequencing (MILLEGEN) as described in example 17.

c) Variant-target yeast strains, screening and sequencing

The yeast strain FYBL2-7B (*MAT a*, *ura3* Δ 851, *trp1* Δ 63, *leu2* Δ 1, *lys2* Δ 202) containing the HIV1_1 target in the yeast reporter vector (pCLS1055, Figure 8) was transformed with one variant, in the kanamycin vector (pCLS1107),
25 cutting the HIV1_4.4 target, using a high efficiency LiAc transformation protocol. Variant-target yeast strains were used as target strains for mating assays as described in example 19. Positives resulting clones were verified by sequencing (MILLEGEN) as described in example 17.

B) Results

Seven variants cleaving HIV1_4.3, were pooled, randomly mutagenized and transformed into yeast. The sequences of the variants subjected to random mutagenesis are described in table XXVI.

5 2232 transformed clones were screened for cleavage against the HIV1_4.3 and HIV1_4.5 DNA targets. A total of 249 positive clones were found to cleave HIV1_4.3, while 12 of them cleaved also the HIV1_4.5 target. Sequencing of the 93 clones showing the strongest activity allowed the identification of 60 novel endonuclease variants. An example of the identified variants is presented in table
10 XXX and in figure 39.

Table XXX: Examples of 10 functional variants displaying strong cleavage activity for HIV1_4.3.

Optimized variants HIV1_4.3	
SEQ ID NO: 189	28Q 36N 38R 40K 44K 68T 70G 75N
SEQ ID NO: 190	28Q 38R 40K 44K 68T 70G 75N 132V
SEQ ID NO: 191	28Q 38R 40K 44K 54L 68A 70S 75N
SEQ ID NO: 192	28Q 38R 40K 44K 54L 68A 70D 75N
SEQ ID NO: 193	28Q 38R 40K 41M 44K 54L 68T 70G 75N
SEQ ID NO: 194	28Q 38R 40K 44K 68T 70G 75N 80K 114T
SEQ ID NO: 195	28Q 38R 40K 44K 68T 70G 72P 75N
SEQ ID NO: 196	28Q 38R 40K 44K 68A 70D 75N 132V 156R
SEQ ID NO: 197	28Q 38R 40K 43L 44K 68T 70G 75N
SEQ ID NO: 198	28Q 38R 40K 44K 68T 70G 75N

15 * Mutations resulting from random mutagenesis are in bold.

The 93 clones showing the highest cleavage activity on target HIV1_4.3 were then mated with a yeast strain that contains (i) the HIV1_4 target in a reporter plasmid (ii) an expression plasmid containing a variant that cleaves the HIV1_4.4 target (I-
20 *CreI* 30H,33M,38A,44N,68Y,70S,75Y,77R or KHSMAS/NYSYR, according to the nomenclature of Table I). After mating with this yeast strain, no clones were found to cleave the HIV1_4 when forming heterodimers with KHSMAS/NYSYR (SEQ ID NO: 177, Table XXIX).

Example 20bis: Improvement of meganucleases cleaving HIV1_4.3 by a second round of random mutagenesis of proteins and assembly with proteins cleaving HIV1_4.4

In order to further improve the activity of the obtained meganucleases, a second round of random mutagenesis was carried out following the same rationale of example 20. For this purpose, four variants cleaving HIV1_4.3 were mutagenized, and variants were screened for cleavage activity of HIV1_4.3 and HIV1_4.5 targets. Additionally the mutants with the strongest activity were screened for cleavage activity of HIV1_4 when co-expressed with a variant cleaving HIV1_4.4.

The materials and methods have previously been described in example 20.

A) Results

Six variants cleaving HIV1_4.3, were pooled, randomly mutagenized and transformed into yeast. The six variants submitted to random mutagenesis correspond to variants described in Table XXX (SEQ ID NO: 189 to 194).

2232 transformed clones were screened for cleavage against the HIV1_4.3 and HIV1_4.5 DNA targets. A total of 377 positive clones were found to cleave HIV1_4.3, while 208 of those cleaved also the HIV1_4.5 target. Sequencing of the 93 clones with the highest activity allowed the identification of 53 novel endonuclease variants. An example of the identified variants is presented in table XXXI and figure 40.

The 93 clones showing cleaving target HIV1_4.3 were then mated with a yeast strain that contains (i) the HIV1_4 target in a reporter plasmid (ii) an expression plasmid containing a variant that cleaves the HIV1_4.4 target (I-CreI 30H,33M,38A,44A,68Y,70S,75Y,77R,155R or KHSMAS/AYSyr +155R, according to the nomenclature of Table I). After mating with this yeast strain, all the 93 clones were found to cleave the HIV1_4. Thus, 93 positives contained proteins able to form heterodimers with KHSMAS/AYSyr +155R (SEQ ID NO: 199) showing cleavage activity on the HIV1_4 target. An example of positives is shown in Figure 41. Sequencing of these 93 positive clones indicates, as mentioned before, that 53 distinct variants were identified. Ten of these 53 variants are presented as an example in Table XXXI.

Table XXXI: Examples of 10 functional variants displaying strong cleavage activity for HIV1_4.3.

Optimized variants HIV1_4.3 (2nd round)	
SEQ ID NO: 200	28Q 38R 40K 44K 68T 70G 80K 100R 114T
SEQ ID NO: 201	28Q 38R 40K 41M 44K 54L 68T 70G 123M 132V
SEQ ID NO: 202	28Q 38R 40K 44K 54L 68A 70S 80K 89S 111R 132V
SEQ ID NO: 203	28Q 38R 40K 44K 68T 70G 80K 114T 162P
SEQ ID NO: 204	28Q 38R 40K 44K 54L 68T 70G 80K 114T
SEQ ID NO: 205	28Q 38R 40K 44K 68T 70G 80K 114T
SEQ ID NO: 206	28Q 38R 40K 44K 54L 57N 68T 70G 132V 159E
SEQ ID NO: 207	28Q 38R 40K 44K 54L 61G 64A 68A 70D 121R 132V
SEQ ID NO: 208	28Q 38R 40K 44K 54L 68T 70G 132V
SEQ ID NO: 209	28Q 35C 38R 40K 43Y 44K 62V 67I 68T 70G 99R 132V

* Mutations resulting from random mutagenesis are in bold.

5 Example 21: Improvement of meganucleases cleaving HIV1_4 by site-directed mutagenesis of proteins cleaving HIV1_4.3 and assembly with proteins cleaving HIV1_4.4

I-*CreI* variants cleaving HIV1_4.3 were also mutagenized by introducing selected amino-acid substitutions in the proteins and screening for more efficient variants cleaving HIV1_4 in combination with a variant cleaving HIV1_4.4.

Six amino-acid substitutions have been found in previous studies to enhance the activity of I-*CreI* derivatives: these mutations correspond to the replacement of Glycine 19 with Serine (G19S), Phenylalanine 54 with Leucine (F54L), Glutamic acid 80 with Lysine (E80K), Phenylalanine 87 with Leucine (F87L), Valine 105 with Alanine (V105A) and Isoleucine 132 with Valine (I132V). These mutations were introduced into the coding sequence of proteins cleaving HIV1_4.3, and the resulting proteins were tested for their ability to induce cleavage of the HIV1_4 target, upon co-expression with a variant cleaving HIV1_4.4, as well as for the ability to cleave targets HIV1_4.3 and HIV1_4.5.

20 A) Material and Methods

a) Site-directed mutagenesis

Site-directed mutagenesis libraries were created by PCR on a pool of chosen variants. For example, to introduce the G19S substitution into the coding sequence of the variants, two separate overlapping PCR reactions were carried out that amplify the 5' end (residues 1-24) or the 3' end (residues 14-167) of the I-*CreI* coding sequence. For both the 5' and 3' end, PCR amplification is carried out

using a primer with homology to the vector (Gal10F 5'-gcaactttagtgtgacacatacagg-3' (SEQ ID NO: 16) or Gal10R 5'-acaaccttgattggagacttgacc-3' (SEQ ID NO: 17)) and a primer specific to the I-CreI coding sequence for amino acids 14-24 that contains the substitution mutation G19S (G19SF 5'-gccggctttgtggactctgacggtagcatcatc-3' (SEQ ID NO: 47) or G19SR 5'-gatgatgctaccgtcagagtccacaaagccggc-3' (SEQ ID NO: 48)).

The same strategy is used with the following pair of oligonucleotides to introduce the mutations leading to the F54L, E80K, F87L, V105A and I132V substitutions in the coding sequences of the variants, respectively:

- * F54LF: 5'-accagcgccgttggtgctggacaaactagtg-3' and F54LR: 5'-cactagttgtccagcagccaacggcgctgggt-3' (SEQ ID NO: 49 and 50);
- * E80KF: 5'-ttaagcaaatcaagccgctgcacaacttctg-3' and E80KR: 5'-caggaagttgtgcagcggcttgatttgcctaa-3' SEQ ID NO: 51 and 52);
- * F87LF: 5'-aagccgctgcacaacctgctgactcaactgcag-3' and F87LR: 5'-ctgcagttgagtcagcaggttggtgcagcggctt-3' SEQ ID NO: 53 and 54);
- * V105AF: 5'-aaacaggcaaacctggctctgaaaattatcgaa-3' and V105AR: 5'-ttcgataattttcagagccaggtttgcctgtt-3' SEQ ID NO: 55 and 56);
- * I132VF: 5'-acctgggtggatcaggttgacgtctgaacgat-3' and I132VR: 5'-atcgttcagagctgcaacctgatccaccaggt-3' SEQ ID NO: 57 and 58).

For each substitution to be introduced, the resulting PCR products contain 33bp of homology with each other. The PCR fragments were purified. The ten PCR fragments were pooled in equimolar amounts to generate a mix containing 50ng of PCR DNA and 75ng of vector DNA (pCLS0542, Figure 9), linearized by digestion with *NcoI* and *EagI*. This mix was used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (*MAT α* , *trp1* Δ 63, *leu2* Δ 1, *his3* Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). Intact coding sequences containing the substitutions are generated *in vivo* by homologous recombination in yeast.

c) Mating of meganuclease expressing clones and screening in yeast

The experimental procedure is as described in example 20.

30 d) Sequencing of variants

The experimental procedure is as described in example 17.

B) Results

A library containing a population harboring the six amino-acid substitutions (Glycine 19 with Serine, Phenylalanine 54 with Leucine, Glutamic acid 80 with Lysine, Phenylalanine 87 with Leucine, Valine 105 with Alanine and Isoleucine 132 with Valine) was constructed on a pool of six variants cleaving HIV1_4.3 (described in Table XXXI, SEQ ID NO:200 to 205).

558 transformed clones were mated with a yeast strain that contains (i) the HIV1_4 target in a reporter plasmid (ii) an expression plasmid containing a variant that cleaves the HIV1_4.4 target (30H,33M,38A,44N,68Y,70S,75Y,77R or KHSMAS/NYSYR, according to the nomenclature of Table I). After mating with this yeast strain, 486 clones were found to cleave the HIV1_4. Thus, 486 positives contained proteins able to form heterodimers with KHSMAS/NYSYR (SEQ ID NO: 177) showing cleavage activity on the HIV1_4 target. An example of positive variants is shown in figure 42.

Sequencing of the 93 clones with the highest cleavage activity on the HIV1_4 target allowed the identification of 34 different endonuclease variants. These 93 clones were also tested for their ability to cleave the HIV1_4.3 and HIV1_4.5 targets. In this case, 71 clones were able to cleave the HIV1_4.3 target, and 69 the HIV1_4.5 target (see Figure 43 for an example). Sequence analysis of these clones showed the presence of 25 different endonuclease variants. Comparison of sequences of the positive clones in all the targets indicated the presence of a total of 40 novel endonuclease variants.

The sequence of ten I-CreI variants cleaving the HIV1_4 target when forming a heterodimer with the KHSMAS/NYSYR variant are listed in Table XXXII.

Table XXXII: Sequences corresponding to the variants cleaving the HIV1_4 target

SEQ ID NO:	I-CreI variants
211	19S 28Q 38R 40K 44K 68T 70G 75N 80K 114T
212	28Q 38R 40K 44K 54L 68T 70G 75N 80K 114T
213	19S 28Q 38R 40K 44K 54L 68T 70G 75N 114T
214	19S 28Q 38R 40K 44K 68T 70G 75N 80K 114T 147A 162P
215	28Q 38R 40K 44K 54L 68T 70G 75N 80K 100R 114T 132V 162P
216	19S 28Q 38R 40K 41M 44K 54L 68T 70G 75N 80K 123M 132V
217	19S 28Q 38R 40K 42A 44K 54L 68T 70G 75N 80K 105A 114T
218	19S 28Q 38R 40K 42S 44K 54L 68T 70G 75N 80K 92H 94Y 105A
219	19S 28Q 38R 40K 41M 44K 54L 68T 70G 75N 80K 114T
220	19S 28Q 38R 40K 44K 68T 70G 75N 123M 132V

* Mutations resulting from site-directed mutagenesis are in bold.

Example 22: Improvement of meganucleases cleaving HIV1_4.4 by random mutagenesis and assembly with proteins cleaving HIV1_4.3

The assembly of I-CreI variants cleaving the palindromic HIV1_4.3 and HIV1_4.4 target to cleave the HIV1_4.2 and HIV1_4 have been previously described in example 19. However, these variants display activity with the HIV1_4.2 target and not with the HIV1_4 target.

As a complement to example 4 we also decided to perform random mutagenesis with variants that cleave HIV1_4.4. Therefore ten variants cleaving HIV1_4.3 were mutagenized, and variants were screened for cleavage activity of HIV1_4.4 and HIV1_4.6 targets. Additionally the mutants with the strongest activity were screened for cleavage activity of HIV1_4 when co-expressed with a variant cleaving HIV1_4.3.

A) Material and Methods

a) Construction of libraries by random mutagenesis

Random mutagenesis was performed on a pool of chosen variants, by PCR using Mn^{2+} . PCR reactions were carried out that amplify the I-CreI coding sequence using the primers preATGCreFor (5'-gcataaattactatacttctatagacacgcaaacacaaatacacagcgcccttgccacc-3'; SEQ ID NO: 24) and ICReIpostRev (5'-ggctcgaggagctcgtctagaggatcgctcgagttatcagtcggccgc-3'; SEQ ID NO: 25). Approximately 25 ng of the PCR product and 75 ng of vector DNA

(pCLS1107, Figure 11) linearized by digestion with *Dra*III and *Ngo*MIV were used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (*MAT* α , *trp1* Δ 63, *leu2* Δ 1, *his3* Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). Expression plasmids containing an
5 intact coding sequence for the I-CreI variant were generated by *in vivo* homologous recombination in yeast.

b) Variant-target yeast strains, screening and sequencing

The yeast strain FYBL2-7B (*MAT* α , *ura3* Δ 851, *trp1* Δ 63, *leu2* Δ 1, *lys2* Δ 202) containing the HIV1_4 target in the yeast reporter vector (pCLS1055,
10 Figure 8) was transformed with variants, in the leucine vector (pCLS0542), cutting the HIV1_4.3 target, using a high efficiency LiAc transformation protocol. Variant-target yeast strains were used as target strains for mating assays as described in example 4. Positives resulting clones were verified by sequencing (MILLEGEN) as described in example 17.

15 **B) Results**

Ten variants cleaving HIV1_4.4 were pooled, randomly mutagenized and transformed into yeast. The sequences of the variants subjected to random mutagenesis are described in table XXXII.

2232 transformed clones were screened for cleavage against the
20 HIV1_4.4 and HIV1_4.6 DNA targets. A total of 210 positive clones were found to cleave HIV1_4.4, while 32 of those also cleaved the HIV1_4.6 target. Sequencing of the 93 clones showing the strongest activity allowed the identification of 65 novel endonuclease variants. An example of the identified variants is presented in table XXXIII and in figure 44.

Table XXXIII: Examples of 10 functional variants displaying strong cleavage activity for HIV1_4.4.

Optimized variants HIV1_4.4	
SEQ ID NO: 199	30H 33M 38A 44A 68Y 70S 75Y 77R 155R
SEQ ID NO: 177	30H 33M 38A 44N 68Y 70S 75Y 77R
SEQ ID NO: 221	30H 33M 38A 44N 68Y 70S 75Y 77R 160R
SEQ ID NO: 222	30H 33M 38A 44N 68Y 70S 75Y 77R 96R
SEQ ID NO: 223	32A 33T 44A 68Y 70S 75Y 77R 98R 129A 158M
SEQ ID NO: 224	26H 30H 33M 38A 44N 68Y 70S 75Y 77R
SEQ ID NO: 225	30H 33M 38A 44N 68Y 70S 75Y 77R 99R
SEQ ID NO: 226	32A 33T 44A 57R 68Y 70S 75Y 77R 125A 132V
SEQ ID NO: 227	30H 33M 38A 44N 68Y 70S 75Y 77R 158R
SEQ ID NO: 228	30H 33M 38A 44N 68Y 70S 75Y 77R 116R

* Mutations resulting from random mutagenesis are in bold.

5

The 93 clones showing the highest cleavage activity on target HIV1_4.4 were then mated with a yeast strain that contains (i) the HIV1_4 target in a reporter plasmid (ii) an expression plasmid containing a variant that cleaves the HIV1_4.3 target (I-*CreI* 28Q,38R,40K,44K,68T,70G,75N +132V or QNSYRK/KTGNI +132V, according to the nomenclature of Table I). After mating with this yeast strain, 90 clones were found to cleave the HIV1_4 target. Thus, 90 positives contained proteins able to form heterodimers with QNSYRK/KTGNI +132V (SEQ ID NO: 190, Table XXX), that showed cleavage activity on the HIV1_4 target. An example of positives is shown in Figure 45. Sequencing of these 90 positive clones indicates that 65 distinct variants were identified. Ten of these 65 variants are presented as an example in Table XXXIII.

Example 23: Improvement of meganucleases cleaving HIV1_4 by site-directed mutagenesis of proteins cleaving HIV1_4.4 and assembly with proteins cleaving HIV1_4.3

Four of the I-*CreI* variants cleaving HIV1_4.4 described in Table XXXVII were mutagenized by introducing selected amino-acid substitutions in the proteins and screening for more efficient variants cleaving HIV1_4 in combination with a variant cleaving HIV1_4.3.

25

Six amino-acid substitutions have been found in previous studies to enhance the activity of I-*CreI* derivatives: these mutations correspond to the replacement of Glycine 19 with Serine (G19S), Phenylalanine 54 with Leucine (F54L),

Glutamic acid 80 with Lysine (E80K), Phenylalanine 87 with Leucine (F87L), Valine 105 with Alanine (V105A) and Isoleucine 132 with Valine (I132V). These mutations were introduced into the coding sequence of proteins cleaving HIV1_4.4, and the resulting proteins were tested for their ability to induce cleavage of the HIV1_4 target, upon co-expression with a variant cleaving HIV1_4.3

A) Material and Methods

a) Site-directed mutagenesis

Site-directed mutagenesis libraries were created by PCR on a pool of chosen variants. For example, to introduce the G19S substitution into the coding sequence of the variants, two separate overlapping PCR reactions were carried out that amplify the 5' end (residues 1-24) or the 3' end (residues 14-167) of the I-*CreI* coding sequence. For both the 5' and 3' end, PCR amplification is carried out using a primer with homology to the vector (Gal10F 5'-gcaacttagtgctgacacatacagg-3' or Gal10R 5'-acaaccttgattggagactgacc-3') and a primer specific to the I-*CreI* coding sequence for amino acids 14-24 that contains the substitution mutation G19S (G19SF 5'-gccggctttgtgactctgacggtagcatcatc-3' (SEQ ID NO: 47) or G19SR 5'-gatgatgctaccgtcagagtccacaaagccggc-3' (SEQ ID NO: 48)). The resulting PCR products contain 33bp of homology with each other. The PCR fragments were purified. Approximately 25ng of each of the two overlapping PCR fragments and 75ng of vector DNA (pCLS1107, Figure 11) linearized by digestion with *DraIII* and *NgoMIV* were used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (*MAT α* , *trp1* Δ 63, *leu2* Δ 1, *his3* Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). Intact coding sequences containing the substitutions are generated *in vivo* by homologous recombination in yeast.

The same strategy is used with the following pair of oligonucleotides to create other libraries containing the F54L, E80K, F87L, V105A and I132V substitutions, respectively:

* F54LF: 5'-accagcgccgttggtgctggacaaactagt-3' and F54LR: 5'-cactagttgtccagcagccaacggcgctgggt-3' (SEQ ID NO: 49 and 50);

* E80KF: 5'-ttaagcaaatcaagccgctgcacaacttctg-3' and E80KR: 5'-caggaagtgtgcagcggcttgatttgcttaa-3' (SEQ ID NO: 51 and 52);

* F87LF: 5'-aagccgctgcacaacctgctgactcaactgcag-3' and F87LR: 5'-ctgcagttgagtcagcaggttgctgcagcggctt-3' SEQ ID NO: 53 and 54);

* V105AF: 5'-aaacaggcaaacctggctctgaaattatcgaa-3' and V105AR: 5'-ttcgataattttcagagccaggtttgcctgttt-3' SEQ ID NO: 55 and 56);

5 * I132VF: 5'-acctgggtggatcaggttgagctctgaacgat-3' and I132VR: 5'-atcgttcagagctgcaacctgatccaccaggt-3' SEQ ID NO: 57 and 58).

c) Mating of meganuclease expressing clones and screening in yeast

The experimental procedure is as described in example 22.

d) Sequencing of variants

10 The experimental procedure is as described in example 17.

B) Results

A library containing a population harboring the six amino-acid substitutions (Glycine 19 with Serine, Phenylalanine 54 with Leucine, Glutamic acid 80 with Lysine, Phenylalanine 87 with Leucine, Valine 105 with Alanine and
15 Isoleucine 132 with Valine) was constructed on a pool of four variants cleaving HIV1_4.4 (see Table XXXIII, SEQ ID NO:199, 177, 221 and 228).

558 transformed clones were mated with a yeast strain that contains (i) the HIV1_4 target in a reporter plasmid (ii) an expression plasmid containing a variant that cleaves the HIV1_4.3 target (28Q,38R,40K,44K,68T,70G,75N or
20 QNSYRK/KTGNI, according to the nomenclature of Table I). After mating with this yeast strain, 16 clones were found to cleave the HIV1_4. Thus, 16 positives contained proteins able to form heterodimers with QNSYRK/KTGNI +132V (SEQ ID NO: 190, Table XXX) showing cleavage activity on the HIV1_4 target. An example of positive variants is shown in figure 46.

25 Sequencing of these positive clones allowed the identification of 10 different endonuclease variants. The clones cleaving the HIV1_4 target were also tested for their ability to cleave the HIV1_4.4 and HIV1_4.6 targets (see Figure 47 for an example). In this case, 15 of the clones were able to cleave the HIV1_4.3 and the HIV1_4.5 targets. Sequence analysis of these clones showed the presence of 10
30 different endonuclease variants. Comparison of sequences of the positive clones in all the targets indicated the presence of a total of 11 novel endonuclease variants.

The sequence of ten I-CreI variants cleaving the HIV1_4 target when forming a heterodimer with the KHSMAS/NYSYR variant are listed in Table XXXIV.

Table XXXIV: Sequences corresponding to the variants cleaving the HIV1_4

5 DNA target

* Mutations resulting from site-directed mutagenesis are in bold.

SEQ ID NO:	Unique mutations, compared to the I-CreI sequence
229	30H 33M 38A 44N 68Y 70S 75Y 77R 92R
230	13Q 19S 30H 33M 38A 44A 68Y 70S 75Y 77R
231	30H 33M 38A 44N 68Y 70S 75Y 77R 132V
232	30H 33M 38A 44N 68Y 70S 75Y 77R
233	13Q 26R 30H 33M 38A 44A 68Y 70S 75Y 77K 92R 112P 132V
234	13Q 26R 30H 33M 38A 44N 68Y 70S 75Y 77R 87L
235	30H 33M 38A 44N 54L 68Y 70S 75Y 77R
236	30H 33M 38A 44N 68Y 70S 75Y 77K 87L 92R 132V
237	28Q 38R 40K 44K 54L 68T 70G 75N 80K 114T
238	13Q 26R 30H 33M 38A 44A 68Y 70S 75Y 77R 92R 160R

Example 24: Strategy for engineering meganucleases cleaving the HIV1_5 target from the HIV1 virus

10 The HIV1_5 target is a 22 bp (non-palindromic) target located in the pol gene of the HIV1 provirus. This target is precisely located at positions 2317-2338 of the HIV-1 pNL4-3 vector (accession number AF324493, Adachi et al., J. Virol., 1986, 59, 284-291), a subtype B infectious molecular clone.

15 The HIV1_5 sequence (SEQ ID NO: 337) is partly a patchwork of the 10TCT_P, 10CTG_P, 5TAG_P and 5_CCT_P targets (Figure 48) which are cleaved by previously identified meganucleases, obtained as described in International PCT Applications WO 2006/097784 and WO 2006/097853; Arnould et al., J. Mol. Biol., 2006, 355, 443-458; Smith et al., Nucleic Acids Res., 2006. Thus, HIV1_5 could be cleaved by combinatorial variants resulting from these previously identified
20 meganucleases.

The 10TCT_P, 10CTG_P, 5TAG_P and 5_CCT_P target sequences are 24 bp derivatives of C1221, a palindromic sequence cleaved by I-CreI (Arnould et al., precited). However, the structure of I-CreI bound to its DNA target suggests that the two external base pairs of these targets (positions -12 and 12) have no impact on
25 binding and cleavage (Chevalier et al., Nat. Struct. Biol., 2001, 8, 312-316; Chevalier

and Stoddard, Nucleic Acids Res., 2001, 29, 3757-3774; Chevalier et al., J. Mol. Biol., 2003, 329, 253-269), and in this study, only positions -11 to 11 were considered. Consequently, the HIV1_5 series of targets were defined as 22 bp sequences instead of 24 bp. HIV1_5 differs from C1221 in the 4 bp central region. According to the structure of the I-CreI protein bound to its target, there is no contact between the 4 central base pairs (positions -2 to 2) and the I-CreI protein (Chevalier et al., Nat. Struct. Biol., 2001, 8, 312-316; Chevalier and Stoddard, Nucleic Acids Res., 2001, 29, 3757-3774; Chevalier et al., J. Mol. Biol., 2003, 329, 253-269). Thus, the bases at these positions should not impact the binding efficiency. However, they could affect cleavage, which results from two nicks at the edge of this region. Thus, the ATAC sequence in -2 to 2 was first substituted with the GTAC sequence from C1221, resulting in target HIV1_5.2 (SEQ ID NO: 338, Figure 48). Then, two palindromic targets, HIV1_5.3 (SEQ ID NO: 339) and HIV1_5.4 (SEQ ID NO: 340), were derived from HIV1_5.2 (Figure 48). Since HIV1_5.3 and HIV1_5.4 are palindromic, they should be cleaved by homodimeric proteins. Two other quasi-palindromic targets were derived from these two, containing the ATAC sequence in -2 to 2 (targets HIV1_5.5 (SEQ ID NO: 341) and HIV1_5.6 (SEQ ID NO: 342), figure 48). Thus, proteins able to cleave HIV1_5.3 and HIV1_5.4 targets or, preferentially, the quasi-palindromic targets as homodimers were first designed (examples 25 and 26) and then co-expressed to obtain heterodimers cleaving HIV1_5 (example 27). Heterodimers cleaving the HIV1_5.2 and HIV1_5 targets could be identified. In order to improve cleavage activity for the HIV1_5 target, a series of variants cleaving HIV1_5.3 and HIV1_5.4 was chosen, and then refined. The chosen variants were subjected to random or site-directed mutagenesis, and used to form novel heterodimers that were screened against the HIV1_5 target (examples 28, 29, 30 and 31). Heterodimers could be identified with an improved cleavage activity for the HIV1_5 target.

Example 25: Identification of meganucleases cleaving HIV1_5.3

This example shows that I-CreI variants can cut the HIV1_5.3 DNA target sequence derived from the left part of the HIV1_5.2 target in a palindromic form (Figure 48).

HIV1_5.3 is similar to 10TCT_P at positions ± 1 , ± 2 , ± 6 , ± 8 , ± 9 , and ± 10 and to 5TAG_P at positions ± 1 , ± 2 , ± 3 , ± 4 , ± 5 and ± 6 . It was hypothesized that positions ± 7 and ± 11 would have little effect on the binding and cleavage activity. Variants able to

cleave the 10TCT_P target were obtained by mutagenesis of I-CreI N75 or D75, at positions 28, 30, 32, 33, 38, 40 and 70, as described previously in Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156. Variants able to cleave 5TAG_P were obtained by mutagenesis on

5 I-CreI N75 at positions 24, 44, 68, 70, 75 and 77 as described in Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2006/097784, WO 2006/097853, WO 2007/060495 and WO 2007/049156.

Both sets of proteins are mutated at position 70. However, the

10 existence of two separable functional subdomains was hypothesized. This implies that this position has little impact on the specificity at bases 10 to 8 of the target. Mutations at positions 24 found in variants cleaving the 5TAG_P target will be lost during the combinatorial process. But it was hypothesized that this will have little impact on the capacity of the combined variants to cleave the HIV1_5.3 target.

15 Therefore, to check whether combined variants could cleave the HIV1_5.3 target, mutations at positions 44, 68, 70, 75 and 77 from proteins cleaving 5TAG_P were combined with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10TCT_P.

A) Material and Methods

20 a) Construction of target vector

The target was cloned as follows: an oligonucleotide corresponding to the HIV1_5.3 target sequence flanked by gateway cloning sequences was ordered from PROLIGO:

5'

TGGCATACAAGTTTGCTCTATTAGGTACCTAATAGAGCCAATCGTCTGTCA

25 3' (SEQ ID NO: 52). The same procedure was followed for cloning the HIV1_5.5 target, using the oligonucleotide: 5'

TGGCATACAAGTTTGCTCTATTAGATACCTAATAGAGCCAATCGTCTGTCA

3' (SEQ ID NO: 53). Double-stranded target DNA, generated by PCR amplification of the single stranded oligonucleotide, was cloned using the Gateway protocol

30 (INVITROGEN) into the yeast reporter vector (pCLS1055, Figure 8). Yeast reporter vector was transformed into *Saccharomyces cerevisiae* strain FYBL2-7B (*MAT* α , *ura3* Δ 851, *trp1* Δ 63, *leu2* Δ 1, *lys2* Δ 202), resulting in a reporter strain.

b) Construction of combinatorial mutants

I-CreI variants cleaving 10TCT_P or 5TAG_P were previously identified, as described in Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156, and Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; International PCT Applications WO 2006/097784 and WO 2006/097853, respectively for the 10TCT_P and 5TAG_P targets. In order to generate I-CreI derived coding sequences containing mutations from both series, separate overlapping PCR reactions were carried out that amplify the 5' end (aa positions 1-43) or the 3' end (positions 39-167) of the I-CreI coding sequence. For both the 5' and 3' end, PCR amplification is carried out using primers (Gal10F 5'-gcaacttagtgctgacacatacagg-3' (SEQ ID NO: 16) or Gal10R 5'-acaaccttgattggagacttgacc-3' (SEQ ID NO: 17)) specific to the vector (pCLS0542, Figure 9) and primers (assF 5'-ctannnttgacctt-3' (SEQ ID NO: 18) or assR 5'-aaaggtcaannntag-3' (SEQ ID NO: 19)), where nnn codes for residue 40, specific to the I-CreI coding sequence for amino acids 39-43. The PCR fragments resulting from the amplification reaction realized with the same primers and with the same coding sequence for residue 40 were pooled. Then, each pool of PCR fragments resulting from the reaction with primers Gal10F and assR or assF and Gal10R was mixed in an equimolar ratio. Finally, approximately 25 ng of each final pool of the two overlapping PCR fragments and 75 ng of vector DNA (pCLS0542, Figure 9) linearized by digestion with *NcoI* and *EagI* were used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (MAT α , trp1 Δ 63, leu2 Δ 1, his3 Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). An intact coding sequence containing both groups of mutations is generated by *in vivo* homologous recombination in yeast.

c) Mating of meganuclease expressing clones and screening in yeast

Screening was performed as described previously (Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458). Mating was performed using a colony gridder (QpixII, GENETIX). Variants were gridded on nylon filters covering YPD plates, using a low gridding density (4-6 spots/cm²). A second gridding process was performed on the same filters to spot a second layer consisting of the reporter-harboring yeast strain. Membranes were placed on solid agar YPD rich medium, and

incubated at 30 °C for one night, to allow mating. Next, filters were transferred to synthetic medium, lacking leucine and tryptophan, with galactose (2 %) as a carbon source, and incubated for five days at 37 °C, to select for diploids carrying the expression and target vectors. After 5 days, filters were placed on solid agarose medium with 0.02 % X-Gal in 0.5 M sodium phosphate buffer, pH 7.0, 0.1 % SDS, 6% dimethyl formamide (DMF), 7 mM β -mercaptoethanol, 1% agarose, and incubated at 37°C, to monitor β -galactosidase activity. Results were analyzed by scanning and quantification was performed using appropriate software.

d) Sequencing of variants

To recover the variant expression plasmids, yeast DNA was extracted using standard protocols and used to transform *E. coli*. Sequencing of variant ORFs was then performed on the plasmids by MILLEGEN SA. Alternatively, ORFs were amplified from yeast DNA by PCR (Akada et al., Biotechniques, 2000, 28, 668-670), and sequencing was performed directly on the PCR product by MILLEGEN SA.

B) Results

I-*CreI* combinatorial variants were constructed by associating mutations at positions 44, 68, 70, 75 and 77 from proteins cleaving 5TAG_P with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10TCT_P on the I-*CreI* scaffold, resulting in a library of complexity 1920. Examples of combinatorial variants are displayed in Table XXXV, none of the variants tested from the combinatorial library produced a positive result. This library was transformed into yeast and 3348 clones (1.7 times the diversity) were screened for cleavage against the HIV1_5.3 and HIV1_5.5 DNA targets. Two positive clones were found (though having weak cleavage activity), which after sequencing turned out to correspond to 2 different novel endonuclease variants (Table XXXVI). These two positives are shown in Figure 49. These two variants display non parental combinations at positions 28, 30, 32, 33, 38, 40 or 44, 68, 70, 75, 77. Such combinations likely result from PCR artifacts during the combinatorial process. Alternatively, the variants may be I-*CreI* combined variants resulting from micro-recombination between two original variants during *in vivo* homologous recombination in yeast.

Table XXXV: Panel of variants* theoretically present in the combinatorial library

Amino acids at positions 44, 68, 70, 75 and 77 (ex : ARNNI stands for A44, R68, N70, N75 and I77)	Amino acids at positions 28, 30, 32, 33, 38 and 40 (ex: KHSSQS stands for K28, H30, S32, S33, Q38 and S40)										
	KASTQS	KCSGQS	KHSCQS	KTSAQS	KNSGTS	KNSTES	KSSSTS	KNSAWS	KQSGQS	KNTCQS	KKSTQS
AYSYK											
ARSNI											
SRSYT											
VERNR											
ARSYT											
TRSYV											
AANNI											
AASYR											
AGNNI											
AHQNI											
NHSYN											
NYSYK											
NYSYV											
NRSYN											
SRSYS											
YRSNV											
ARDNI											
ARSYI											
NRSYI											
ANANI											
ARHDI											
DNSNI											
TYSYK											
ARSYT											

*Only 264 out of the 1920 combinations are displayed. None of them were identified in the positive clones.

Table XXXVI: I-CreI variants with additional mutations capable of cleaving the HIV1_5.3 DNA target.

Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77 of the I-CreI variants (ex: KRSRES/TYSNI stands for K28, R30, S32, R33, E38, S40/ T44, Y68, S70, N75 and I77)	SEQ ID NO:
KNSCYS/AYQNI	241
KNSCAS/NHSYN +80K	242

10 **Example 26: Making of meganucleases cleaving HIV1_5.4**

This example shows that I-CreI variants can cleave the HIV1_5.4 DNA target sequence derived from the right part of the HIV1_5.2 target in a palindromic form (Figure 4).

HIV1_5.4 is similar to 5CCT_P at positions ± 1 , ± 2 , ± 3 , ± 4 , ± 5 and ± 8 and to 10CTG_P at positions ± 1 , ± 2 , ± 3 , ± 4 , ± 8 , ± 9 and ± 10 . It was hypothesized that positions ± 6 , ± 7 and ± 11 would have little effect on the binding and cleavage activity. Variants able to cleave 5CCT_P were obtained by mutagenesis of I-CreI N75 at positions 44, 68, 70, 75 and 77, as described previously (Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2006/097784, WO 2006/097853, WO 2007/060495 and WO 2007/049156). Variants able to cleave the 10TGG_P target were obtained by mutagenesis of I-CreI N75 or D75, at positions 28, 30, 32, 33, 38, 40 and 70, as described previously in Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156.

Both sets of proteins are mutated at position 70. However, the existence of two separable functional subdomains was hypothesized. This implies that this position has little impact on the specificity at bases 10 to 8 of the target.

Therefore, to check whether combined variants could cleave the HIV1_5.4 target, mutations at positions 44, 68, 70, 75 and 77 from proteins cleaving 5CCT_P were combined with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10CTG_P.

A) Material and Methods

20 a) Construction of target vector

The experimental procedure is as described in example 2, with the exception that different oligonucleotides corresponding to the HIV1_5.4 and HIV1_5.6 targets. The oligonucleotide used for the HIV1_5.4 target was: 5' TGGCATAACAAGTTTATCTGCTCCTGTACAGGAGCAGATCAATCGTCTGTCA 3' (SEQ ID NO: 243), and 5' TGGCATAACAAGTTTATCTGCTCCTATACAGGAGCAGATCAATCGTCTGTCA 3' (SEQ ID NO: 244) for HIV1_5.6 target.

b) Construction of combinatorial variants

I-CreI variants cleaving 10CTG_P or 5CCT_P were previously identified, as described in Smith *et al.* Nucleic Acids Res., 2006, 34, e149; International PCT Applications WO 2007/060495 and WO 2007/049156, and Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458; International PCT Applications WO

2006/097784 and WO 2006/097853, respectively for the 10CTG_P and 5CCT_P targets. In order to generate I-CreI derived coding sequences containing mutations from both series, separate overlapping PCR reactions were carried out that amplify the 5' end (aa positions 1-43) or the 3' end (positions 39-167) of the I-CreI coding sequence. For both the 5' and 3' end, PCR amplification is carried out using primers (Gal10F 5'-gcaactttagtgctgacacatacagg-3' (SEQ ID NO: 16) or Gal10R 5'-acaaccttgattggagacttgacc-3' (SEQ ID NO: 17)) specific to the vector (pCLS1107, Figure 11) and primers (assF 5'-ctannnttgacctt-3' (SEQ ID NO: 18) or assR 5'-aaaggtcaannntag-3' (SEQ ID NO: 19), where nnn codes for residue 40, specific to the I-CreI coding sequence for amino acids 39-43. The PCR fragments resulting from the amplification reaction realized with the same primers and with the same coding sequence for residue 40 were pooled. Then, each pool of PCR fragments resulting from the reaction with primers Gal10F and assR or assF and Gal10R was mixed in an equimolar ratio. Finally, approximately 25 ng of each final pool of the two overlapping PCR fragments and 75 ng of vector DNA (pCLS1107, Figure 11) linearized by digestion with DraIII and NgoMIV were used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (MAT α , trp1 Δ 63, leu2 Δ 1, his3 Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). An intact coding sequence containing both groups of mutations is generated by *in vivo* homologous recombination in yeast.

c) Mating of meganuclease expressing clones and screening in yeast

Screening was performed as described previously (Arnould *et al.*, J. Mol. Biol., 2006, 355, 443-458). Mating was performed using a colony gridded (QpixII, GENETIX). Variants were gridded on nylon filters covering YPD plates, using a low gridding density (4-6 spots/cm²). A second gridding process was performed on the same filters to spot a second layer consisting of the reporter-harboring yeast strain. Membranes were placed on solid agar YPD rich medium, and incubated at 30 °C for one night, to allow mating. Next, filters were transferred to synthetic medium, lacking tryptophan, adding G418, with galactose (2 %) as a carbon source, and incubated for five days at 37 °C, to select for diploids carrying the expression and target vectors. After 5 days, filters were placed on solid agarose medium with 0.02 % X-Gal in 0.5 M sodium phosphate buffer, pH 7.0, 0.1 % SDS,

6% dimethyl formamide (DMF), 7 mM β -mercaptoethanol, 1% agarose, and incubated at 37°C, to monitor β -galactosidase activity. Results were analyzed by scanning and quantification was performed using appropriate software. Positives resulting clones were verified by sequencing (MILLEGEN) as described in example 2.

5 B) Results

I-*CreI* combinatorial variants were constructed by associating mutations at positions 44, 68, 70, 75 and 77 from proteins cleaving 5CCT_P with the 28, 30, 32, 33, 38 and 40 mutations from proteins cleaving 10CTG_P on the I-*CreI* scaffold, resulting in a library of complexity 1600. Examples of combinatorial variants are displayed in Table XXXXI. This library was transformed into yeast and 3348 clones (2 times the diversity) were screened for cleavage against the HIV1_5.4 and HIV1_5.6 DNA targets. A total of 10 positive clones were found to cleave HIV1_5.4. Sequencing of these 10 clones allowed the identification of 9 novel endonuclease variants, which are represented in Table XXXVII. Examples of positives are shown in Figure 50. The sequence of several of the variants identified display non parental combinations at positions 28, 30, 32, 33, 38, 40 or 44, 68, 70, 75, 77 as well as additional mutations (Table XXXVIII, SEQ ID 246, 247, 251, 252 and 253). Such variants likely result from PCR artifacts during the combinatorial process. Alternatively, the variants may be I-*CreI* combined variants resulting from micro-recombination between two original variants during *in vivo* homologous recombination in yeast.

Table XXXVII: Panel of variants* theoretically present in the combinatorial library

Amino acids at positions 44, 68, 70, 75 and 77 (ex : ARNNI stands for A44, R68, N70, N75 and I77)	Amino acids at positions 28, 30,32, 33, 38 and 40 (ex: KHSSQS stands for K28, H30, S32, S33, Q38 and S40)										
	KDSRSS	KWSTQS	KASSQS	KPSGQS	KDSRSS	KQSGQS	KQSTQS	KNTCQS	KSSNQS	KSSTQS	KTSGQS
KASNI											
QASET											
KESDK											
KTGNI											
KRSDA											
KYSNI											
KASDK											
KGTNI											
KTSDI											
KTSDR									+		
DASKR											
KESDR											
KYSYQ											
RASNN											
RYSNN			+						+		
KNTNI											
KRGNI											
KDSNR											
RASNI											
KYSYI											
RYSNI											
KESNR											
RRSND											
KNSNI											

* Only 264 out of the 1600 combinations are displayed.

- 5 + indicates that a functional combinatorial variant cleaving the HIV1_5.4 target was found among the identified positives.

Table XXXVIII: I-CreI variants capable of cleaving the HIV1_5.4 target.

Amino acids at positions 28, 30, 32, 33, 38, 40 / 44, 68, 70, 75 and 77 of the I-CreI variants (ex: KRSRES/TYSNI stands for K28, R30, S32, R33, E38, S40/ T44, Y68, S70, N75 and I77)	SEQ ID NO:
KASSQS/RYSNN	245
KQSGQS/KYSNT	246
KQSTQS/KYSNQ	247
KSSNQS/KTSDR	248
KSSNQS/KTSDR	249
KSSNQS/KTSDR +132V	250
KSSTQS/KYSNQ	251
KTSGQS/KYSNR +151A	252
KNSSQS/KYSNI	253

Example 27: Making of meganucleases cleaving HIV1_5.2 and HIV1_5

I-*CreI* variants able to cleave each of the palindromic HIV1_5.2 derived targets (HIV1_5.3 and HIV1_5.4) were identified in example 25 and example 26. Pairs of such variants (one cutting HIV1_5.3 and one cutting HIV1_5.4) were co-expressed in yeast. Upon co-expression, there should be three active molecular species, two homodimers, and one heterodimer. It was assayed whether the heterodimers that should be formed, cut the HIV1_5.2 and the non palindromic HIV1_5 targets.

A) Materials and Methods10 a) Construction of target vector

The experimental procedure is as described in example 2, with the exception that an oligonucleotide corresponding to the HIV1_5.2 target sequence: 5'TGGCATAACAAGTTTGCTCTATTAGGTACAGGAGCAGATCAATCGTCTGTC A3' (SEQ ID NO: 254) or the HIV1_5 target sequence:

15 5'TGGCATAACAAGTTTGCTCTATTAGATAACAGGAGCAGATCAATCGTCTGTC A 3'

(SEQ ID NO: 255) was used.

b) Co-expression of variants

Yeast DNA was extracted from variants cleaving the HIV1_5.4 target in the pCLS1107 expression vector using standard protocols and was used to transform *E. coli*. The resulting plasmid DNA was then used to transform yeast strains expressing a variant cutting the HIV1_5.3 target in the pCLS0542 expression vector. Transformants were selected on synthetic medium lacking leucine and containing G418.

25 c) Mating of meganucleases coexpressing clones and screening in yeast

Mating was performed using a colony gridder (QpixII, Genetix). Variants were gridded on nylon filters covering YPD plates, using a low gridding density (4-6 spots/cm²). A second gridding process was performed on the same filters to spot a second layer consisting of different reporter-harboring yeast strains for each target. Membranes were placed on solid agar YPD rich medium, and incubated at 30°C for one night, to allow mating. Next, filters were transferred to synthetic medium, lacking leucine and tryptophan, adding G418, with galactose (2 %) as a

carbon source, and incubated for five days at 37 °C, to select for diploids carrying the expression and target vectors. After 5 days, filters were placed on solid agarose medium with 0.02 % X-Gal in 0.5 M sodium phosphate buffer, pH 7.0, 0.1 % SDS, 6% dimethyl formamide (DMF), 7mM β -mercaptoethanol, 1% agarose, and incubated at 37°C, to monitor β -galactosidase activity. Results were analyzed by scanning and quantification was performed using appropriate software.

B) Results

Co-expression of variants cleaving the HIV1_5.4 target (9 variants chosen among those described in Table XXXVIII) and the two variants cleaving the HIV1_5.3 target (described in Table XXXVI) resulted in cleavage of the HIV1_5.2 target in one of the cases (Figure 51). Nevertheless, this combination was not able to cut the HIV1_5 natural target, that differs from the HIV1_5.2 sequence by 2 bp at positions 1 and 2 (Figure 48). The functional combination cleaving the HIV1_5.2 target correspond to mutants KNSCYS/AYQNI (SEQ ID 241, cleaving HIV1_5.3) and KTSGQS/KYSDR +151A (SEQ ID 252, cleaving HIV1_5.4)

Example 28: Improvement of meganucleases cleaving HIV1_5.3 by random mutagenesis and assembly with proteins cleaving HIV1_5.4

I-CreI variants able to cleave the HIV1_5.3 have been identified in example 25. Since these two variants show a weak activity, and only one of them is able to cleave the HIV1_5.2 target when assembled with a meganuclease cleaving the HIV1_5.4, these two variants were mutagenized, and the clones generated were screened for cleavage activity of HIV1_5.3 and HIV1_5.5 targets. Additionally the mutants with the strongest activity were screened for cleavage activity of HIV1_5 when co-expressed with a variant cleaving HIV1_5.4. According to the structure of the I-CreI protein bound to its target, there is no contact between the 4 central base pairs (positions -2 to 2) and the I-CreI protein (Chevalier *et al.*, Nat. Struct. Biol., 2001, 8, 312-316; Chevalier and Stoddard, Nucleic Acids Res., 2001, 29, 3757-3774; Chevalier *et al.*, J. Mol. Biol., 2003, 329, 253-269). Thus, it is difficult to rationally choose a set of positions to mutagenize, and mutagenesis was performed on the whole protein. Random mutagenesis results in high complexity libraries. Therefore, to limit the complexity of the variant libraries to be tested, only one of the two components of the heterodimers cleaving HIV1_5 was mutagenized.

Thus, in a first step, proteins cleaving HIV1_5.3 were mutagenized and their homodimeric cleavage activity was determined, and in a second step, it was assessed whether they could cleave HIV1_5 when co-expressed with a protein cleaving HIV1_5.4.

5 A) Material and Methods

a) Construction of libraries by random mutagenesis

Random mutagenesis was performed on a pool of chosen variants, by PCR using Mn^{2+} . PCR reactions were carried out that amplify the I-CreI coding sequence using the primers preATGCreFor (5'-
 10 gcataaattactatacttctatagacacgcaaacacaaatacacagcggccttgccacc-3'; SEQ ID NO: 24) and ICReIpostRev (5'-ggctcgaggagctcgtctagaggatcgctcgagttatcagtcggccgc-3'; SEQ ID NO: 25), which are common to the pCLS0542 (Figure 9) and pCLS1107 (Figure 11) vectors. Approximately 25 ng of the PCR product and 75 ng of vector DNA (pCLS0542) linearized by digestion with *NcoI* and *EagI* were used to transform the
 15 yeast *Saccharomyces cerevisiae* strain FYC2-6A (*MAT α* , *trp1* Δ 63, *leu2* Δ 1, *his3* Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). Expression plasmids containing an intact coding sequence for the I-CreI variant were generated by *in vivo* homologous recombination in yeast.

20 b) Mating of meganuclease expressing clones and screening in yeast

Experiment were performed as previously described in example 25. Positive resulting clones were verified by sequencing (MILLEGEN) as described in example 25.

c) Variant-target yeast strains, screening and sequencing

25 The yeast strain FYBL2-7B (*MAT a*, *ura3* Δ 851, *trp1* Δ 63, *leu2* Δ 1, *lys2* Δ 202) containing the HIV1_5 target in the yeast reporter vector (pCLS1055, Figure 8) was transformed with one variant, in the kanamycin vector (pCLS1107), cutting the HIV1_5.4 target, using a high efficiency LiAc transformation protocol. Variant-target yeast strains were used as target strains for mating assays as described
 30 in example 27. Positives resulting clones were verified by sequencing (MILLEGEN) as described in example 25.

B) Results

Two variants cleaving HIV1_5.3, were pooled, randomly mutagenized and transformed into yeast. The sequences of the variants subjected to random mutagenesis are described in table XXXVI.

2232 transformed clones were screened for cleavage against the HIV1_5.3 and HIV1_5.5 DNA targets. A total of 20 positive clones were found to cleave HIV1_5.3, while none of those cleaved the HIV1_5.5 target. Sequencing of the 20 clones allowed the identification of 13 novel endonuclease variants. An example of these variants is presented in table XXXIX and in figure 52.

Table XXXIX: Examples of 10 functional improved variants displaying cleavage activity for HIV1_5.3.

Optimized variants HIV1_5.3	
SEQ ID NO: 256	33C 38Y 44A 68Y 70Q 75N 89A
SEQ ID NO: 257	33C 38A 44N 68H 70S 75Y 77N 80K 103S
SEQ ID NO: 258	33C 38Y 44A 60E 68Y 70Q 75N 103D
SEQ ID NO: 259	33C 38A 44N 68H 70S 75Y 77N 80K
SEQ ID NO: 260	33C 38A 44N 59A 68H 70S 75Y 77N 80K
SEQ ID NO: 261	33C 38Y 43L 44A 68Y 70Q 75N
SEQ ID NO: 262	33C 38Y 44A 54L 68Y 70Q 75N 117G
SEQ ID NO: 263	33C 38Y 44A 68Y 70Q 75N
SEQ ID NO: 264	6S 30S 33C 38Y 44A 68H 70S 75Y 77N 80K
SEQ ID NO: 265	33C 38Y 44A 68Y 70Q 72Y 75N 107R

* Mutations resulting from random mutagenesis are in bold

The 20 clones showing cleavage activity on target HIV1_5.3 were also mated with a yeast strain that contains (i) the HIV1_5 target in a reporter plasmid (ii) an expression plasmid containing a variant that cleaves the HIV1_5.4 target (SEQ ID 252; I-CreI 30T,33G,44K,68Y,70S,77R +151A or KTSGQS/KYSDR +151A, according to the nomenclature of Table I). After mating with this yeast strain, no clones were found to cleave the HIV1_5 target.

Example 28bis: Improvement of meganucleases cleaving HIV1_5.3 by a second round of random mutagenesis and assembly with proteins cleaving HIV1_5.4

In order to further improve the activity of the obtained meganucleases, a second round of random mutagenesis was carried out following the same rationale of example 28. For this purpose, ten variants cleaving HIV1_5.3 were mutagenized, and variants were screened for cleavage activity of HIV1_5.3 and HIV1_5.5 targets.

Additionally, the mutants with the strongest activity were screened for cleavage activity of HIV1_5 when co-expressed with a variant cleaving HIV1_5.4.

The materials and methods have previously been described in example 28.

A) Results

5 Ten variants cleaving HIV1_5.3, were pooled, randomly mutagenized and transformed into yeast. The variants submitted to random mutagenesis correspond to variants described in Table XXXIX (SEQ ID NO: 256 to 265).

2232 transformed clones were screened for cleavage against the HIV1_5.3 and HIV1_5.5 DNA targets. A total of 80 positive clones were found to
10 cleave HIV1_5.3, while 25 of those cleaved also the HIV1_5.5 target. Sequencing of the 80 clones allowed the identification of 39 novel endonuclease variants. An example of the identified variants is presented in table XXXX and figure 53.

The 80 clones showing cleavage activity on target HIV1_5.3 were then mated with a yeast strain that contains (i) the HIV1_5 target in a reporter plasmid
15 (ii) an expression plasmid containing a variant that cleaves the HIV1_5.4 target (I-*CreI* 30S,33N,44K,68Y,70S,77R +103T or KSSNQS/KYSDR +103T, according to the nomenclature of Table I). After mating with this yeast strain, 4 clones were found to cleave the HIV1_5. Thus, 4 positives contained proteins able to form heterodimers with KSSNQS/KYSDR +103T (SEQ ID NO: 276) showing cleavage activity on the
20 HIV1_5 target. An example of positives is shown in Figure 54. These 4 variants are presented as an example in Table XXXX (SEQ ID 266 to 269).

Table XXXX: Examples of 10 functional variants displaying strong cleavage activity for HIV1_5.3.

Optimized variants HIV1_5.3 (2nd round)	
SEQ ID NO: 266	33C 38Y 44A 50R 60E 68Y 70Q 75N 79T 85R 103D
SEQ ID NO: 267	33C 38A 44N 68H 70S 75Y 77N 80K 161P
SEQ ID NO: 268	24F 33C 38Y 44A 68Y 70Q 72Y 75N 107R 153Y 163G 164G
SEQ ID NO: 269	33C 38Y 44A 66H 68Y 70Q 72Y 75N 108V
SEQ ID NO: 270	6S 11Q 30S 33C 38Y 44A 68Y 70Q 75N 89A
SEQ ID NO: 271	33C 38A 44N 68H 70S 75Y 77N 80K 103S 132V
SEQ ID NO: 272	33C 38Y 44A 68Y 70Q 75N 89A
SEQ ID NO: 273	33C 38Y 44A 68Y 70Q 72Y 75N
SEQ ID NO: 274	33C 38Y 44A 60E 68Y 70Q 75N 103D 157V
SEQ ID NO: 275	33C 38Y 44A 68Y 70Q 75N 89A 114T 151A

* Mutations resulting from random mutagenesis are in bold.

Example 29: Improvement of meganucleases cleaving HIV1_5 by site-directed mutagenesis of proteins cleaving HIV1_5.3 and assembly with proteins cleaving HIV1_5.4

Three of the I-*CreI* variants cleaving HIV1_5.3 described in
 5 Table XXXX were mutagenized by introducing selected amino-acid substitutions in the proteins and screening for more efficient variants cleaving HIV1_5 in combination with a variant cleaving HIV1_5.4.

Six amino-acid substitutions have been found in previous studies to enhance the activity of I-*CreI* derivatives: these mutations correspond to the
 10 replacement of Glycine 19 with Serine (G19S), Phenylalanine 54 with Leucine (F54L), Glutamic acid 80 with Lysine (E80K), Phenylalanine 87 with Leucine (F87L), Valine 105 with Alanine (V105A) and Isoleucine 132 with Valine (I132V). These mutations were introduced into the coding sequence of proteins cleaving HIV1_5.3, and the resulting proteins were tested for their ability to induce cleavage of the
 15 HIV1_5 target, upon co-expression with a variant cleaving HIV1_5.4, as well as for the ability to cleave targets HIV1_5.3 and HIV1_5.5.

A) Material and Methods

a) Site-directed mutagenesis

Site-directed mutagenesis libraries were created by PCR on a
 20 pool of chosen variants. For example, to introduce the G19S substitution into the coding sequence of the variants, two separate overlapping PCR reactions were carried out that amplify the 5' end (residues 1-24) or the 3' end (residues 14-167) of the I-*CreI* coding sequence. For both the 5' and 3' end, PCR amplification is carried out using a primer with homology to the vector (Gal10F 5'-gcaactttagtgctgacacatacagg-3' (SEQ ID NO: 16) or Gal10R 5'-acaaccttgattggagacttgacc-3' (SEQ ID NO: 17)) and a
 25 primer specific to the I-*CreI* coding sequence for amino acids 14-24 that contains the substitution mutation G19S (G19SF 5'-gccggctttgtggactctgacggtagcatcatc-3' (SEQ ID NO: 47) or G19SR 5'-gatgatgctaccgtcagagtccacaaagccggc-3' (SEQ ID NO: 48)).

The same strategy is used with the following pair of oligonucleotides to introduce the
 30 mutations leading to the F54L, E80K, F87L, V105A and I132V substitutions in the coding sequences of the variants, respectively:

- * F54LF: 5'-accagcgccgttggtgctggacaaactagt-3' and F54LR: 5'-cactagttgtccagcagccaacggcgctgggt-3' (SEQ ID NO: 49 and 50);
- * E80KF: 5'-ttaagcaaaatcaagccgctgcacaacttcctg-3' and E80KR: 5'-caggaagttgtgcagcggttgatttgcctaa-3' SEQ ID NO: 51 and 52);
- 5 * F87LF: 5'-aagccgctgcacaacctgctgactcaactgcag-3' and F87LR: 5'-ctgcagttgagtcagcaggttgctgcagcggt-3' SEQ ID NO: 53 and 54);
- * V105AF: 5'-aaacaggcaaacctggctctgaaaattatcgaa-3' and V105AR: 5'-ttcgataattttcagagccaggtttgcctgtt-3' SEQ ID NO: 55 and 56);
- * I132VF: 5'-acctgggtggatcaggttgagctctgaacgat-3' and I132VR: 5'-atcggttcagagctgcaacctgatccaccaggt-3' SEQ ID NO: 57 and 58).
- 10

For each substitution to be introduced, the resulting PCR products contain 33bp of homology with each other. The PCR fragments were purified. The ten PCR fragments were pooled in equimolar amounts to generate a mix containing 50ng of PCR DNA and 75ng of vector DNA (pCLS0542, Figure 9), linearized by digestion with *NcoI* and *EagI*. This mix was used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (*MAT α* , *trp1* Δ 63, *leu2* Δ 1, *his3* Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). Intact coding sequences containing the substitutions are generated *in vivo* by homologous recombination in yeast.

15

20 c) Mating of meganuclease expressing clones and screening in yeast

The experimental procedure is as described in example 28.

d) Sequencing of variants

The experimental procedure is as described in example 25.

B) Results

25 A library containing a population harboring the six amino-acid substitutions (Glycine 19 with Serine, Phenylalanine 54 with Leucine, Glutamic acid 80 with Lysine, Phenylalanine 87 with Leucine, Valine 105 with Alanine and Isoleucine 132 with Valine) was constructed on a pool of three variants cleaving HIV1_5.3 (SEQ ID NO: 266, 269 and 270; described in Table XXXX). 558

30 transformed clones were screened for cleavage against the HIV1_5.3 and HIV1_5.5 DNA targets. A total of 450 positive clones were found to cleave HIV1_5.3, while

435 of those cleaved also the HIV1_5.5 target. An example of positive variants is shown in figure 55.

The 558 transformed clones were also mated with a yeast strain that contains (i) the HIV1_5 target in a reporter plasmid (ii) an expression plasmid containing a variant that cleaves the HIV1_5.4 target (I-CreI 30S,33N,44K,68Y,70S,77R +103T or KSSNQS/KYSDR +103T, according to the nomenclature of Table I). After mating with this yeast strain, 444 clones were found to cleave the HIV1_5. Thus, 444 positives contained proteins able to form heterodimers with KSSNQS/KYSDR +103T (SEQ ID NO: 276) showing cleavage activity on the HIV1_5 target. An example of positive clones is shown in Figure 56.

Sequencing of the 93 clones with the highest cleavage activity on the HIV1_5 target allowed the identification of 50 different endonuclease variants.

The sequence of ten I-CreI variants cleaving the HIV1_5 target when forming a heterodimer with the KSSNQS/KYSDR +103T variant are listed in Table XXXXI.

Table XXXXI: Examples of 10 functional variants displaying strong cleavage activity for HIV1_5

Optimized variants HIV1_5.3	
SEQ ID NO: 277	6S 30S 33C 38Y 44A 50R 60E 68Y 70Q 75N 79T 85R 108V
SEQ ID NO: 278	33C 38Y 44A 50R 60E 68Y 70Q 75N 79T 85R 103D
SEQ ID NO: 279	19S 33C 38Y 44A 50R 60E 68Y 70Q 75N 79T 85R 108V
SEQ ID NO: 280	19S 33C 38Y 44A 68Y 70Q 75N 79T 85R 105A
SEQ ID NO: 281	6S 11Q 19S 30S 33C 38Y 44A 68Y 70Q 75N 89A 105A 132V
SEQ ID NO: 282	33C 38Y 44A 50R 60E 68Y 70Q 75N 79T 85R 105A
SEQ ID NO: 283	33C 38Y 44A 66H 68Y 70Q 73G 75N 89A 105A
SEQ ID NO: 284	19S 33C 38Y 44A 50R 60E 68Y 70Q 75N 79T 85R 103D
SEQ ID NO: 285	6S 11Q 19S 33C 38Y 44A 66H 68Y 70Q 72Y 75N 103D 108V
SEQ ID NO: 286	33C 38Y 44A 68Y 70Q 75N 79T 85R 105A

* Mutations resulting from site directed mutagenesis are in bold.

Example 30: Improvement of meganucleases cleaving HIV1_5.4 by random mutagenesis and assembly with proteins cleaving HIV1_5.3

As a complement to example 29 we also decided to perform random mutagenesis with variants that cleave HIV1_5.4. The variants generated were screened for their cleavage activity on targets HIV1_5.4 and HIV1_5.6; and the

mutagenized proteins cleaving HIV1_5.4 were then tested to determine if they could efficiently cleave HIV1_5 when co-expressed with a protein cleaving HIV1_5.3.

A) Material and Methods

a) Construction of libraries by random mutagenesis

5 Random mutagenesis was performed on a pool of chosen variants, by PCR using Mn^{2+} . PCR reactions were carried out that amplify the I-CreI coding sequence using the primers preATGCreFor (5'-gcataaattactatacttctatagacacgcaaacacaaatacacagcggccttgccacc-3'; SEQ ID NO: 24) and ICrelpostRev (5'-ggctcgaggagctcgtctagaggatcgctcgagttatcagtcggccgc-3'; SEQ ID
10 NO: 25). Approximately 25 ng of the PCR product and 75 ng of vector DNA (pCLS1107, Figure 11) linearized by digestion with *Dra*III and *Ngo*MIV were used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (*MAT* α , *trp1* Δ 63, *leu2* Δ 1, *his3* Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). Expression plasmids containing an
15 intact coding sequence for the I-CreI variant were generated by *in vivo* homologous recombination in yeast.

b) Variant-target yeast strains, screening and sequencing

The yeast strain FYBL2-7B (*MAT* α , *ura3* Δ 851, *trp1* Δ 63, *leu2* Δ 1, *lys2* Δ 202) containing the HIV1_5 target in the yeast reporter vector (pCLS1055,
20 Figure 8) was transformed with variants, in the leucine vector (pCLS0542), cutting the HIV1_5.3 target, using a high efficiency LiAc transformation protocol. Variant-target yeast strains were used as target strains for mating assays as described in example 27. Positives resulting clones were verified by sequencing (MILLEGEN) as described in example 25.

25 B) Results

Nine variants cleaving HIV1_5.4 were pooled, randomly mutagenized and transformed into yeast. The sequences of the variants subjected to random mutagenesis are described in Table XXXVIII.

2232 transformed clones were screened for cleavage against the
30 HIV1_5.4 and HIV1_5.6 DNA targets. A total of 53 positive clones were found to cleave HIV1_5.4, while 6 of those also cleaved the HIV1_5.6 target. Sequencing of the 53 clones showing the strongest activity allowed the identification of 42 novel

endonuclease variants. An example of the identified variants is presented in Table XXXXII and in figure 57.

5 **Table XXXXII: Examples of 10 functional variants displaying strong cleavage activity for HIV1_5.4.**

Optimized variants HIV1_5.4	
SEQ ID NO: 276	30S 33N 44K 68Y 70S 77R 103T
SEQ ID NO: 288	30S 33N 44R 54I 68Y 70S 75N 77Q 124V
SEQ ID NO: 289	30A 33S 44R 66H 68Y 70S 75N 77N 89S
SEQ ID NO: 290	33S 43L 44K 54L 68Y 70S 75Y 92R
SEQ ID NO: 291	30S 33N 44R 56N 68Y 70S 75N 77N 132V
SEQ ID NO: 292	33S 43L 44K 54L 68Y 70S 75Y 82R 132V
SEQ ID NO: 293	33S 44K 68Y 70S 75N 77V
SEQ ID NO: 294	30Q 33T 44K 68Y 70S 77R 83S 151A 159R
SEQ ID NO: 295	30T 33G 44K 66F 68Y 70S 77R 151A
SEQ ID NO: 296	33S 44K 54L 68Y 70S 75N

* Mutations resulting from random mutagenesis are in bold.

10 The 53 positive clones showing the highest cleavage activity on target HIV1_5.4 were then mated with a yeast strain that contains (i) the HIV1_5 target in a reporter plasmid (ii) an expression plasmid containing a variant that cleaves the HIV1_5.3 target (I-CreI 33C,38Y,44A,68Y,70Q,75N +89A or KNSCYS/AYQNI +89A, according to the nomenclature of Table I; SEQ ID 256). After mating with this yeast strain, no clones were found to cleave the HIV1_5 target.

15 **Example 30bis: Improvement of meganucleases cleaving HIV1_5 by a second round of random mutagenesis of proteins cleaving HIV1_5.4 and assembly with proteins cleaving HIV1_5.3**

20 In order to further improve the activity of the obtained meganucleases, a second round of random mutagenesis was carried out following the same rationale of example 30. For this purpose, six variants cleaving HIV1_5.4 were mutagenized, and variants were screened for cleavage activity of HIV1_5.4 and HIV1_5.6 targets. Additionally the mutants were screened for cleavage activity of HIV1_5 when co-expressed with a variant cleaving HIV1_5.3.

The materials and methods have previously been described in example 30.

A) Results

Six variants cleaving HIV1_5.4, were pooled, randomly mutagenized and transformed into yeast. The six variants submitted to random mutagenesis correspond to variants described in Table XXXXII (SEQ ID NO: 276 and 288 to 292).

2232 transformed clones were screened for cleavage against the HIV1_5.4 and HIV1_5.6 DNA targets. A total of 21 positive clones were found to cleave HIV1_5.4, while 9 of those cleaved also the HIV1_5.6 target. Sequencing of the 21 clones allowed the identification of 16 novel endonuclease variants. An example of the identified variants is presented in Table XXXXIII and figure 58.

The 21 positive clones showing cleavage activity on target HIV1_5.4 were then mated with a yeast strain that contains (i) the HIV1_5 target in a reporter plasmid (ii) an expression plasmid containing a variant that cleaves the HIV1_5.3 target (*I-CreI* 33C,38Y,44A,68Y,70Q,75N +89A or KNSCYS/AYQNI +89A, according to the nomenclature of Table I; SEQ ID 256). After mating with this yeast strain, no clones were found to cleave the HIV1_5 target.

Table XXXXIII: Examples of 10 functional variants displaying strong cleavage activity for HIV1_5.4.

Optimized variants HIV1_5.4 (2 nd round)	
SEQ ID NO: 297	6S 33S 44R 54I 68Y 70S 75N 77Q 124V 158R 163T
SEQ ID NO: 298	30S 33N 44K 68Y 70S 77R 103T
SEQ ID NO: 299	30S 33N 44K 68Y 70S 77R 103T 142R 160E
SEQ ID NO: 300	30S 33N 44R 54I 68Y 70S 75N 77Q 124V
SEQ ID NO: 301	33S 43L 44K 45M 54L 66H 68Y 70S 75N 77N 89S
SEQ ID NO: 302	2Y 16L 30S 33S 44R 66H 68Y 70S 75N 77N 82E 89S 103S 147A
SEQ ID NO: 303	33S 43L 44K 54L 68Y 70S 75Y 92R 114Y
SEQ ID NO: 304	33S 43L 44K 54L 68Y 70S 75Y 92R
SEQ ID NO: 305	33S 43L 44K 54L 68Y 70S 75Y 92R 153Y
SEQ ID NO: 306	30A 33S 44R 64A 66H 68Y 70S 75N 77N 89S 103D 128R 146V 151A

* Mutations resulting from random mutagenesis are in bold.

Example 31: Improvement of meganucleases cleaving HIV1_5 by site-directed mutagenesis of proteins cleaving HIV1_5.4 and assembly with proteins cleaving HIV1_5.3

Two of the *I-CreI* variants cleaving HIV1_5.4 described in Table XXXXIII were mutagenized by introducing selected amino-acid substitutions in the proteins and screening for more efficient variants cleaving HIV1_5.4 and

HIV1_5.6, as well as for cleavage of the HIV1_5 target when in combination with a variant cleaving HIV1_5.3.

Six amino-acid substitutions have been found in previous studies to enhance the activity of I-*CreI* derivatives: these mutations correspond to the replacement of Glycine 19 with Serine (G19S), Phenylalanine 54 with Leucine (F54L), Glutamic acid 80 with Lysine (E80K), Phenylalanine 87 with Leucine (F87L), Valine 105 with Alanine (V105A) and Isoleucine 132 with Valine (I132V).

A) Material and Methods

a) Site-directed mutagenesis

Site-directed mutagenesis libraries were created by PCR on a pool of chosen variants. For example, to introduce the G19S substitution into the coding sequence of the variants, two separate overlapping PCR reactions were carried out that amplify the 5' end (residues 1-24) or the 3' end (residues 14-167) of the I-*CreI* coding sequence. For both the 5' and 3' end, PCR amplification is carried out using a primer with homology to the vector (Gal10F 5'-gcaactttagtgctgacacatacagg-3' (SEQ ID NO: 16) or Gal10R 5'-acaaccttgattggagacttgacc-3' (SEQ ID NO: 17)) and a primer specific to the I-*CreI* coding sequence for amino acids 14-24 that contains the substitution mutation G19S (G19SF 5'-gccggcttggactctgacggtagcatcatc-3' (SEQ ID NO: 47) or G19SR 5'-gatgatgctaccgtcagagtcacaaagccggc-3' (SEQ ID NO: 48)).

The same strategy is used with the following pair of oligonucleotides to introduce the mutations leading to the F54L, E80K, F87L, V105A and I132V substitutions in the coding sequences of the variants, respectively:

- * F54LF: 5'-accagcgccgttgctgctggacaaactagtg-3' and F54LR: 5'-cactagttgtccagcagccaacggcgctgggt-3' (SEQ ID NO: 49 and 50);
- * E80KF: 5'-ttaagcaaatcaagccgctgcacaacttcttg-3' and E80KR: 5'-caggaagtgtgcagcggttgatttgcttaa-3' SEQ ID NO: 51 and 52);
- * F87LF: 5'-aagccgctgcacaacctgctgactcaactgcag-3' and F87LR: 5'-ctgcagttgagtcagcaggttgctgcagcggtt-3' SEQ ID NO: 53 and 54);
- * V105AF: 5'-aacaggcaaacctggctctgaaattatcgaa-3' and V105AR: 5'-ttcgataattttcagagccaggttgctgcttt-3' SEQ ID NO: 55 and 56);
- * I132VF: 5'-acctgggtggatcaggttgacgtctgaacgat-3' and I132VR: 5'-atcggtcagagctgcaacctgatccaccaggt-3' SEQ ID NO: 57 and 58).

For each substitution to be introduced, the resulting PCR products contain 33bp of homology with each other. The PCR fragments were purified. The ten PCR fragments were pooled in equimolar amounts to generate a mix containing 50ng of PCR DNA and 75ng of vector DNA (pCLS0542, Figure 9), linearized by digestion with *NcoI* and *EagI*. This mix was used to transform the yeast *Saccharomyces cerevisiae* strain FYC2-6A (*MAT α* , *trp1* Δ 63, *leu2* Δ 1, *his3* Δ 200) using a high efficiency LiAc transformation protocol (Gietz and Woods, Methods Enzymol., 2002, 350, 87-96). Intact coding sequences containing the substitutions are generated *in vivo* by homologous recombination in yeast.

10 c) Mating of meganuclease expressing clones and screening in yeast

The experimental procedure is as described in example 28.

d) Sequencing of variants

The experimental procedure is as described in example 25.

B) Results

15 A library containing a population harboring the six amino-acid substitutions (Glycine 19 with Serine, Phenylalanine 54 with Leucine, Glutamic acid 80 with Lysine, Phenylalanine 87 with Leucine, Valine 105 with Alanine and Isoleucine 132 with Valine) was constructed on a pool of two variants cleaving HIV1_5.4 (SEQ ID NO: 297 and 299; described in Table XXXXVII). 558 transformed clones were screened for cleavage against the HIV1_5.4 and HIV1_5.6 DNA targets. A total of 378 positive clones were found to cleave HIV1_5.4, while 321 of those cleaved also the HIV1_5.6 target. An example of positive variants is shown in figure 59.

25 The 558 transformed clones were also mated with a yeast strain that contains (i) the HIV1_5 target in a reporter plasmid (ii) an expression plasmid containing a variant that cleaves the HIV1_5.3 target (I-CreI 33C,38Y,44A,68Y,70Q,75N +89A or KNSCYS/AYQNI +89A, according to the nomenclature of Table I). After mating with this yeast strain, 137 clones were found to cleave the HIV1_5. Thus, 137 positives contained proteins able to form
30 heterodimers with KNSCYS/AYQNI +89A (SEQ ID NO: 256) showing cleavage activity on the HIV1_5 target. An example of positives is shown in figure 60.

Sequencing of the 93 clones with the highest cleavage activity on the HIV1_5 target allowed the identification of 48 different endonuclease variants.

The sequence of ten I-CreI variants cleaving the HIV1_5 target when forming a heterodimer with the KNSCYS/AYQNI +89A variant are listed in
5 Table XXXXIV.

Table XXXXIV: Examples of 10 functional variants displaying strong cleavage activity for HIV1_5

Optimized variants HIV1_5.4	
SEQ ID NO: 307	19S 33S 44R 54I 68Y 70S 75N 77Q 124V 158R 163T
SEQ ID NO: 308	6S 19S 30S 33N 44K 68Y 70S 77R 103T 142R 160E
SEQ ID NO: 309	19S 30S 33N 44K 68Y 70S 77R 103T 132V 142R 160E
SEQ ID NO: 310	30S 31R 33S 44R 54I 68Y 70S 75N 77Q 124V 158R 163T
SEQ ID NO: 311	30S 33N 44K 54I 68Y 70S 75N 77Q 158R 163T 164T
SEQ ID NO: 312	6S 19S 30S 33N 44K 68Y 70S 77R 80K 89A 103T 124V 158R 163T
SEQ ID NO: 313	6S 19S 30S 33N 44K 68Y 70S 77R 103T 158R 163T
SEQ ID NO: 314	30S 44K 68Y 70S 77R 103T 160E
SEQ ID NO: 315	19S 30S 33N 44K 68Y 70S 77R 103T 142R 160E
SEQ ID NO: 316	6S 19S 33S 44R 54I 68Y 70S 75N 77Q 105A 124V 131R 158R 163T

10 * Mutations resulting from site directed mutagenesis are in bold.

Claims

1. An I-*CreI* variant, characterized in that at least one of the two I-*CreI* monomers has at least two substitutions, one in each of the two functional subdomains of the LAGLIDADG core domain situated from positions 26 to 40 and 44 to 77 of I-*CreI*, said variant being able to cleave a DNA target sequence from a genomically integrated provirus (GIP), and being obtainable by a method comprising at least the steps of:

(a) constructing a first series of I-*CreI* variants having at least one substitution in a first functional subdomain of the LAGLIDADG core domain situated from positions 26 to 40 of I-*CreI*,

(b) constructing a second series of I-*CreI* variants having at least one substitution in a second functional subdomain of the LAGLIDADG core domain situated from positions 44 to 77 of I-*CreI*,

(c) selecting and/or screening the variants from the first series of step (a) which are able to cleave a mutant I-*CreI* site wherein at least one of (i) the nucleotide triplet in positions -10 to -8 of the I-*CreI* site has been replaced with the nucleotide triplet which is present in positions -10 to -8 of said DNA target sequence from the GIP and (ii) the nucleotide triplet in positions +8 to +10 has been replaced with the reverse complementary sequence of the nucleotide triplet which is present in position -10 to -8 of said DNA target sequence from the GIP,

(d) selecting and/or screening the variants from the second series of step (b) which are able to cleave a mutant I-*CreI* site wherein at least one of (i) the nucleotide triplet in positions -5 to -3 of the I-*CreI* site has been replaced with the nucleotide triplet which is present in positions -5 to -3 of said DNA target sequence from the GIP and (ii) the nucleotide triplet in positions +3 to +5 has been replaced with the reverse complementary sequence of the nucleotide triplet which is present in position -5 to -3 of said DNA target sequence from the GIP,

(e) selecting and/or screening the variants from the first series of step (a) which are able to cleave a mutant I-*CreI* site wherein at least one of (i) the nucleotide triplet in positions +8 to +10 of the I-*CreI* site has been replaced with the nucleotide triplet which is present in positions +8 to +10 of said DNA target sequence from the GIP and (ii) the nucleotide triplet in positions -10 to -8 has been replaced

with the reverse complementary sequence of the nucleotide triplet which is present in position +8 to +10 of said DNA target sequence from the GIP,

(f) selecting and/or screening the variants from the second series of step (b) which are able to cleave a mutant I-*CreI* site wherein at least one of (i) the
5 nucleotide triplet in positions +3 to +5 of the I-*CreI* site has been replaced with the nucleotide triplet which is present in positions +3 to +5 of said DNA target sequence from the GIP and (ii) the nucleotide triplet in positions -5 to -3 has been replaced with the reverse complementary sequence of the nucleotide triplet which is present in position +3 to +5 of said DNA target sequence from the GIP,

10 (g) combining in a single variant, the mutation(s) in positions 26 to 40 and 44 to 77 of two variants from step (c) and step (d), to obtain a novel homodimeric I-*CreI* variant which cleaves a sequence wherein (i) the nucleotide triplet in positions -10 to -8 is identical to the nucleotide triplet which is present in positions -10 to -8 of said DNA target sequence from the GIP, (ii) the nucleotide
15 triplet in positions +8 to +10 is identical to the reverse complementary sequence of the nucleotide triplet which is present in positions -10 to -8 of said DNA target sequence from the GIP, (iii) the nucleotide triplet in positions -5 to -3 is identical to the nucleotide triplet which is present in positions -5 to -3 of said DNA target sequence from the GIP and (iv) the nucleotide triplet in positions +3 to +5 is identical to the
20 reverse complementary sequence of the nucleotide triplet which is present in positions -5 to -3 of said DNA target sequence from the GIP, and/or

(h) combining in a single variant, the mutation(s) in positions 26 to 40 and 44 to 77 of two variants from step (e) and step (f), to obtain a novel homodimeric I-*CreI* variant which cleaves a sequence wherein (i) the nucleotide
25 triplet in positions +8 to +10 of the I-*CreI* site has been replaced with the nucleotide triplet which is present in positions +8 to +10 of said DNA target sequence from the GIP and (ii) the nucleotide triplet in positions -10 to -8 is identical to the reverse complementary sequence of the nucleotide triplet in positions +8 to +10 of said DNA target sequence from the GIP, (iii) the nucleotide triplet in positions +3 to +5 is
30 identical to the nucleotide triplet which is present in positions +3 to +5 of said DNA target sequence from the GIP, (iv) the nucleotide triplet in positions -5 to -3 is

identical to the reverse complementary sequence of the nucleotide triplet which is present in positions +3 to +5 of said DNA target sequence from the GIP,

(i) combining the variants obtained in steps (g) and (h) to form heterodimers, and

5 (j) selecting and/or screening the heterodimers from step (i) which are able to cleave said DNA target sequence from the GIP.

2. The variant of claim 1, wherein said variant may be obtained by a method comprising the additional steps of:

10 (k) selecting heterodimers from step (j) and constructing a third series of variants having at least one substitution in at least one of the monomers in said selected heterodimers,

(l) combining said third series variants of step (k) and screening the resulting heterodimers for altered cleavage activity against said DNA target from the GIP.

15 3. The variant of claim 2, wherein in said step (k) said at least one substitution are introduced by site directed mutagenesis in a DNA molecule encoding said third series of variants, and/or by random mutagenesis in a DNA molecule encoding said third series of variants.

20 4. The variants of claim 2 or 3, wherein steps (k) and (l) are repeated at least two times and wherein the heterodimers selected in step (k) of each further iteration are selected from heterodimers screened in step (l) of the previous iteration which showed increased cleavage activity against said DNA target from the GIP.

25 5. The variant of any one of claims 1 to 4, wherein said substitution(s) in the subdomain situated from positions 44 to 77 of I-CreI are in positions 44, 68, 70, 75 and/or 77.

6. The variant of any one of claims 1 to 5, wherein said substitution(s) in the subdomain situated from positions 26 to 40 of I-CreI are in positions 26, 28, 30, 32, 33, 38 and/or 40.

30 7. The variant of any one of claims 1 to 6, which comprises one or more substitutions in positions 137 to 143 of I-CreI that modify the specificity of the variant towards the nucleotide in positions ± 1 to 2, ± 6 to 7 and/or ± 11 to 12 of the target site in the GIP.

8. The variant of any one of claims 1 to 7, which comprises one or more substitutions on the entire *I-CreI* sequence that improve the binding and/or the cleavage properties of the variant towards said DNA target sequence from the GIP.

9. The variant of anyone of claims 1 to 8, wherein said DNA target
5 sequence is from the HIV1 provirus.

10. The variant according to claim 9, wherein said DNA target is located in an element of the HIV1 provirus selected from the group: an essential gene, a regulatory sequence for an essential gene, an essential structural sequence.

11. The variant of claim 9 or 10 wherein said DNA target is selected
10 from the group consisting of the sequences SEQ ID NO: 319 to 342.

12. The variant of claim 9 or 10 consisting of the sequences SEQ ID NO: 1-13; SEQ ID NO: 26-46; SEQ ID NO: 59-85; SEQ ID NO: 88-94; SEQ ID NO: 97-165; SEQ ID NO: 168-174; SEQ ID NO: 177-186; SEQ ID NO: 189-238; SEQ ID NO: 241-242; SEQ ID NO: 245-253; SEQ ID NO: 256-316.

13. The variant of any one of claims 1 to 12, wherein said
15 substitutions are replacement of the initial amino acids with amino acids selected in the group consisting of A, D, E, G, H, K, N, P, Q, R, S, T, Y, C, W, L and V.

14. The variant of any one of claims 1 to 13, which is a heterodimer, resulting from the association of a first and a second monomer having different
20 mutations in positions 26 to 40 and 44 to 77 of *I-CreI*, said heterodimer being able to cleave a non-palindromic DNA target sequence from the GIP.

15. The variant of claim 14, which is an obligate heterodimer, wherein the first and the second monomer, respectively, further comprises the D137R mutation and the R51D mutation.

16. The variant of claim 14 or 15, which is an obligate heterodimer, wherein the first monomer further comprises the K7R, E8R, E61R, K96R and L97F or K7R, E8R, F54W, E61R, K96R and L97F mutations and the second monomer further
25 comprises the K7E, F54G, L58M and K96E or K7E, F54G, K57M and K96E mutations.

17. A single-chain chimeric meganuclease comprising two
30 monomers or core domains of one or two variant(s) of anyone of claims 1 to 13, or a combination of both.

18. The single-chain meganuclease of claim 17 which comprises the first and the second monomer as defined in anyone of claims 1 to 13, connected by a peptide linker.

19. A polynucleotide fragment encoding the variant of anyone of
5 claims 1 to 15 or the single-chain chimeric meganuclease of claim 17 or claim 18.

20. An expression vector comprising at least one polynucleotide fragment of claim 19.

21. The vector of claim 20, which includes a targeting construct comprising a sequence to be introduced flanked by sequences sharing homologies
10 with the regions surrounding said DNA target sequence is from the GIP.

22. The vector of claim 20 or 21, wherein said sequence to be introduced is a sequence which inactivates the GIP.

23. The vector of claim 22, wherein the sequence which inactivates the GIP comprises in the 5' to 3' orientation: a first transcription termination sequence
15 and a marker cassette including a promoter, the marker open reading frame and a second transcription termination sequence, and said sequence interrupts the transcription of the coding sequence.

24. The vector of any one of claims 21 to 23, wherein said sequence sharing homologies with the regions surrounding DNA target sequence is a fragment
20 of the GIP comprising sequences upstream and downstream of the cleavage site, so as to allow the deletion of coding sequences flanking the cleavage site.

25. A host cell which is modified by a polynucleotide of claim 18 or a vector of anyone of claims 20 to 24.

26. A non-human transgenic animal which is modified by a
25 polynucleotide of claim 19 or a vector of anyone of claims 20 to 24.

27. A transgenic plant which is modified by a polynucleotide of claim 19 or a vector of anyone of claims 20 to 24

28. Use of at least one variant of anyone of claims 1 to 16, or at least one single-chain chimeric meganuclease of claim 17 or 18, or at least one vector
30 according to anyone of claims 20 to 24, for genome engineering, for non-therapeutic purposes.

29. Use of at least one variant of anyone of claims 1 to 16, or at least one single-chain chimeric meganuclease of claim 17 or 18, or at least one vector according to anyone of claims 20 to 24, for the preparation of a medicament for preventing, improving or curing a pathological condition caused by the GIP.

- 5 30. The use of claim 28 or 29, wherein said variant or said single-chain chimeric meganuclease, or said vector is associated with a targeting DNA construct as defined in anyone of claims 20 to 23.

1/60

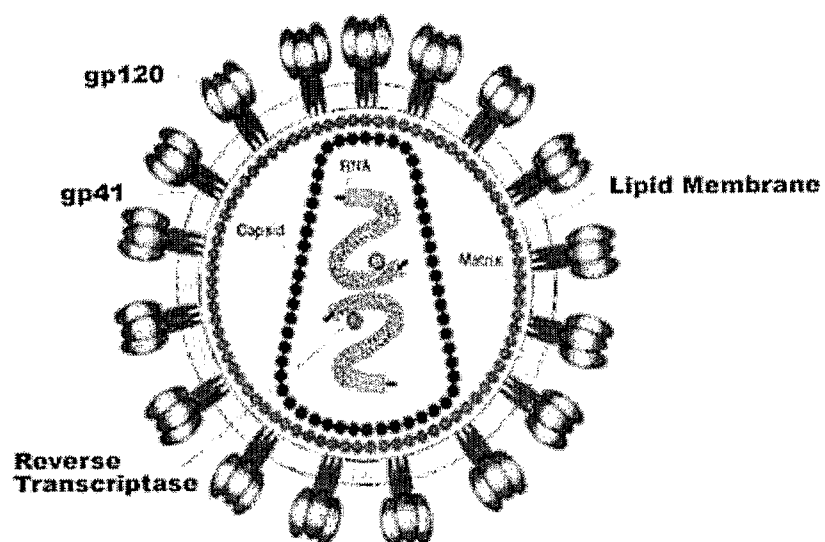


Figure 1

2/60

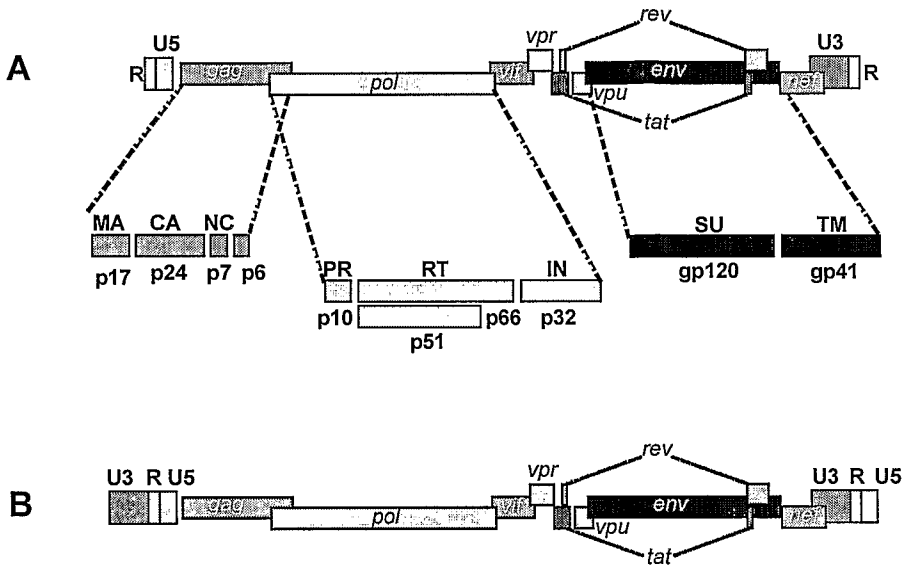


Figure 2

3/60

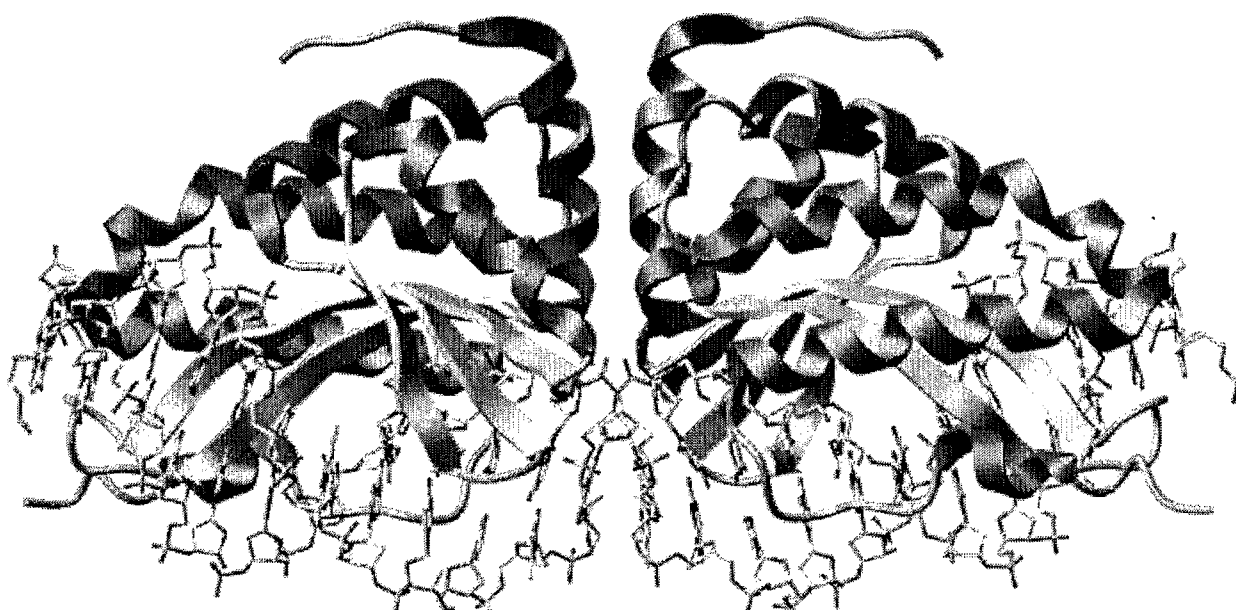


Figure 3

4/60

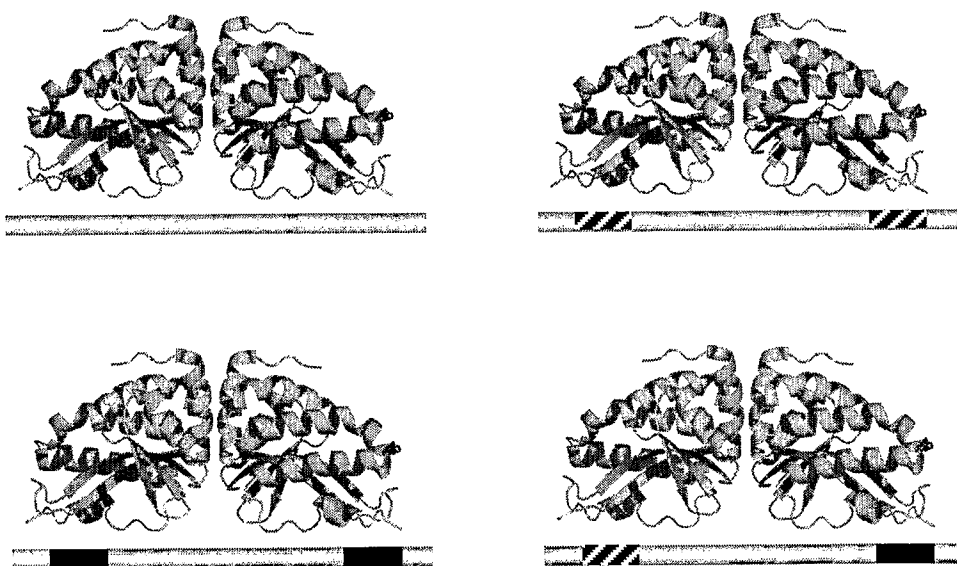


Figure 4

5/60

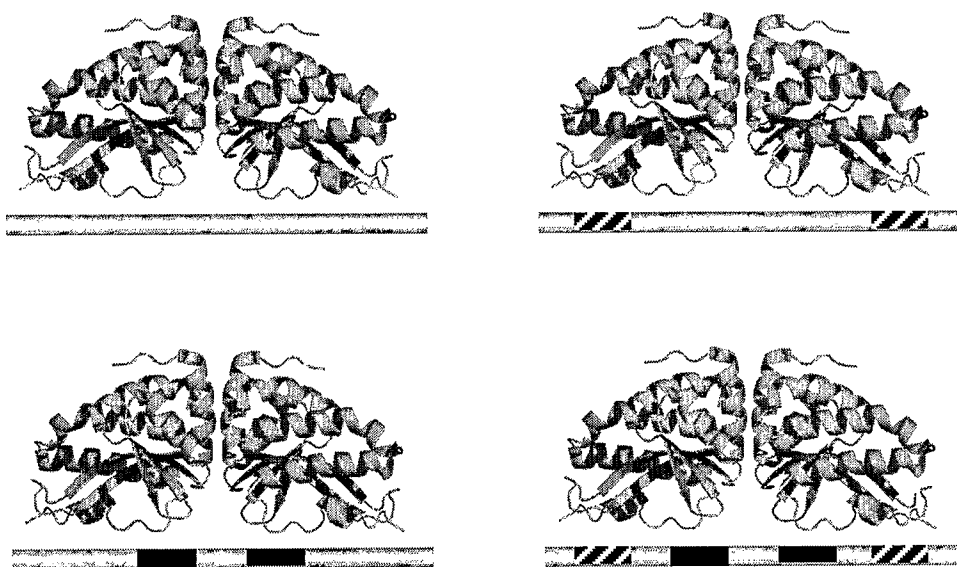


Figure 5

6/60

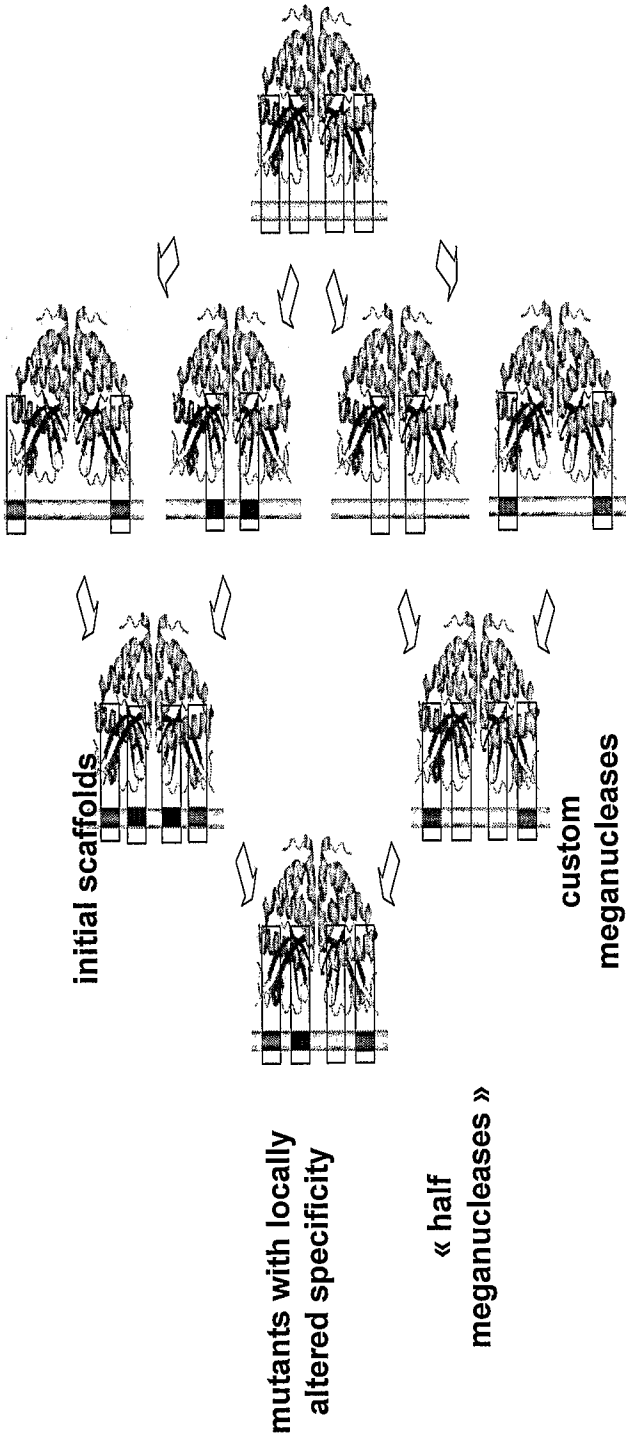


Figure 6

7/60

		⁻¹⁰		⁻⁵	⁻²	⁺²	⁺⁵		⁺¹⁰		
HIV1_1	C	AGA	AC	TAC	ACAC	CAG	GG	CCA	G	(SEQ ID NO:319)	
HIV1_1.2	C	AGA	AC	TAC	GTAC	CAG	GG	CCA	G	(SEQ ID NO:320)	
HIV1_1.3	C	AGA	AC	TAC	GTAC	GTA	GT	TCT	G	(SEQ ID NO:321)	
HIV1_1.4	C	TGG	CC	CTG	GTAC	CAG	GG	CCA	G	(SEQ ID NO:322)	
HIV1_1.5	C	AGA	AC	TAC	ACAC	GTA	GT	TCT	G	(SEQ ID NO:323)	
HIV1_1.6	C	TGG	CC	CTG	ACAC	CAG	GG	CCA	G	(SEQ ID NO:324)	

Figure 7

8/60

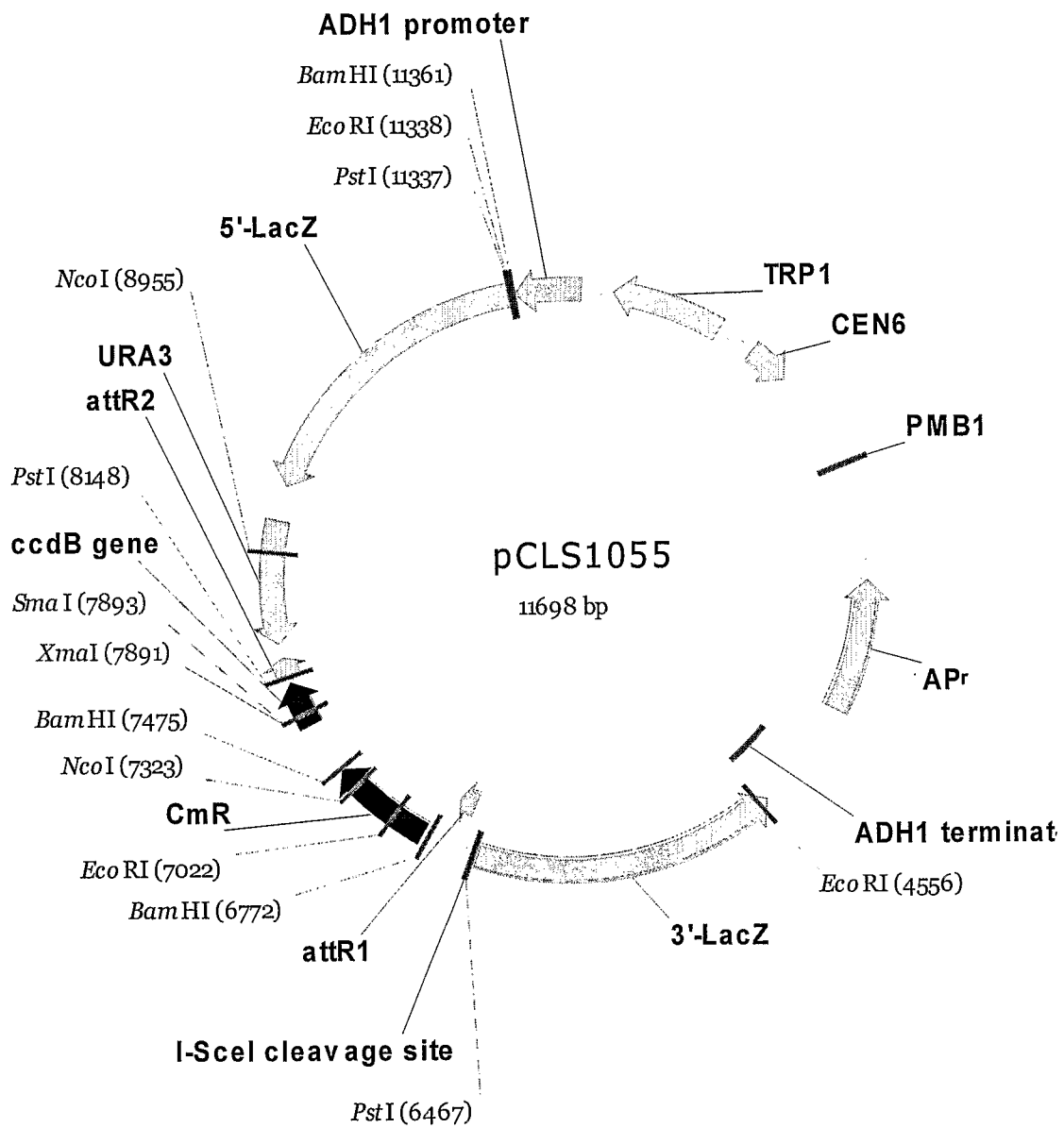


Figure 8

9/60

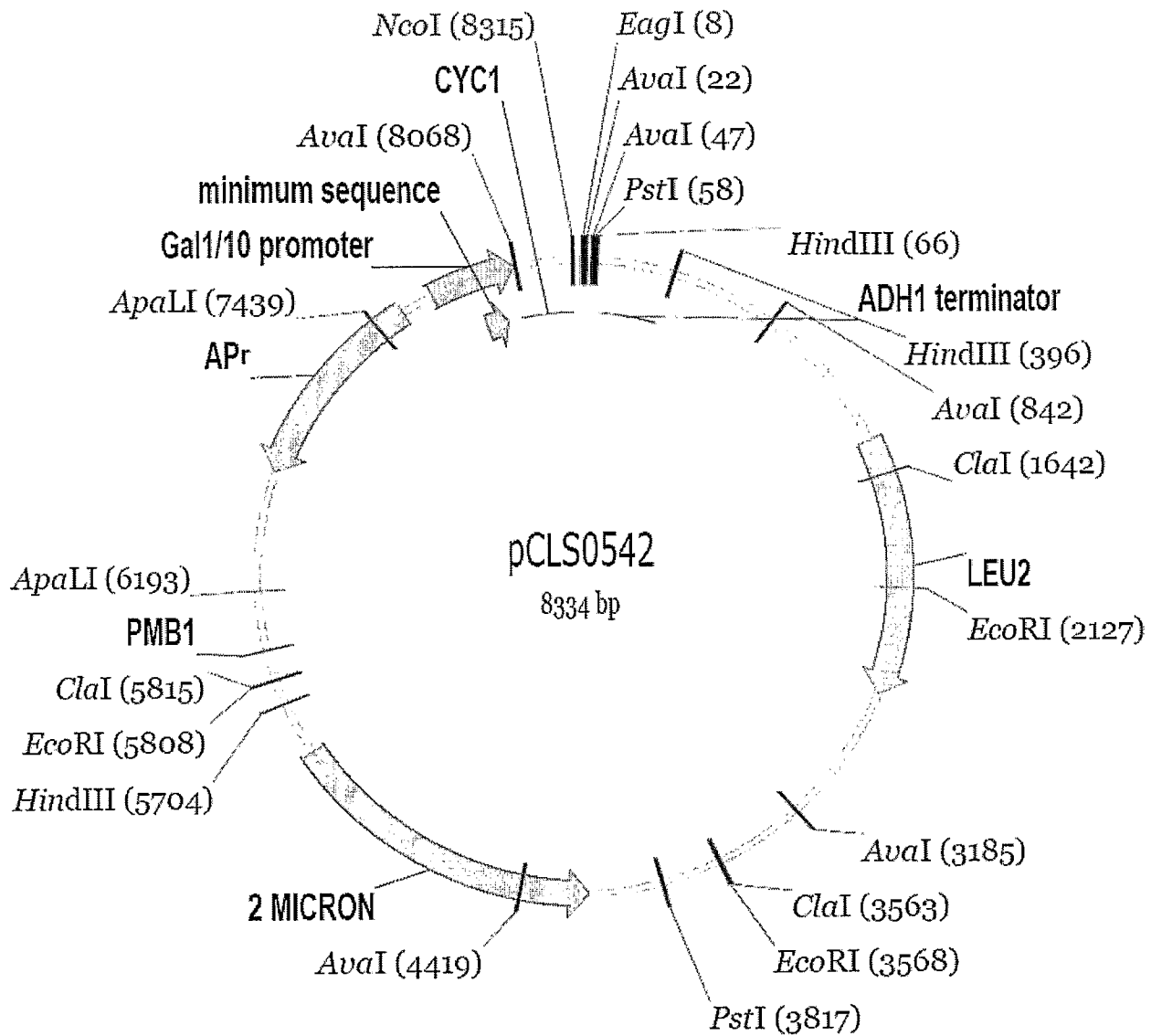


Figure 9

10/60

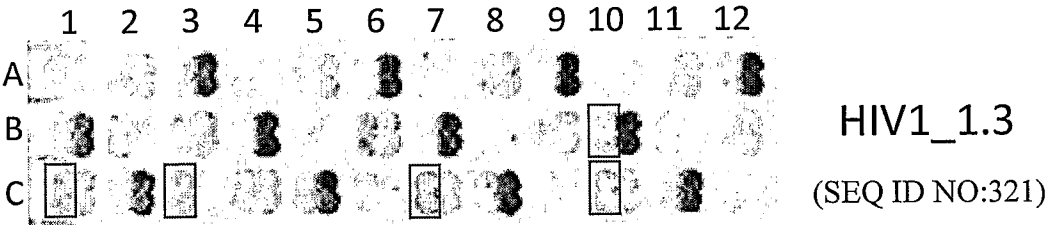


Figure 10

11/60

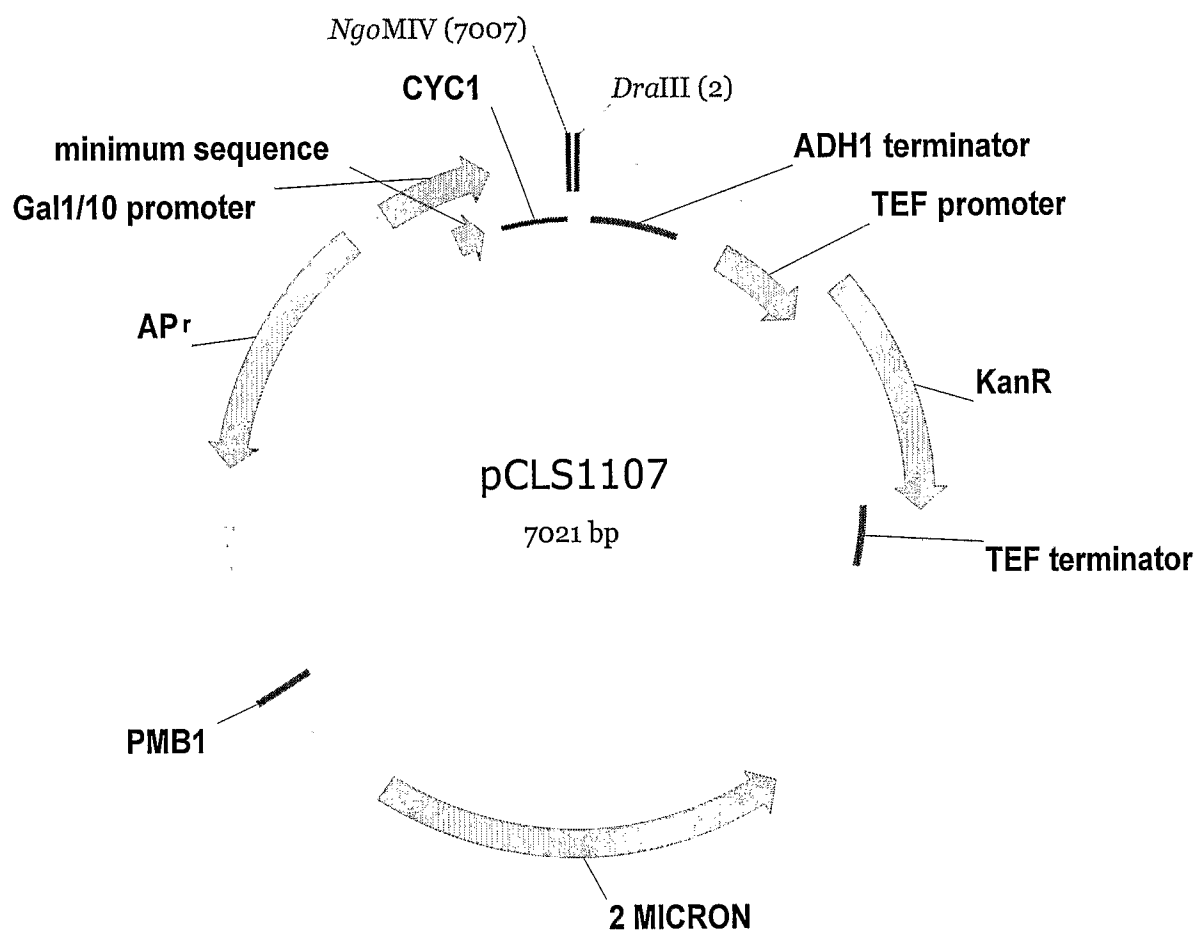


Figure 11

12/60

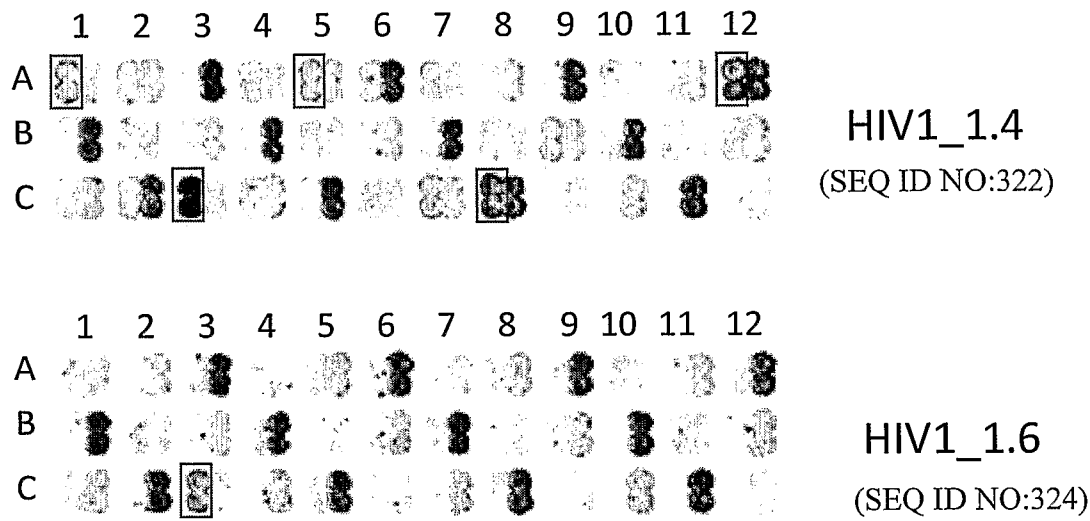
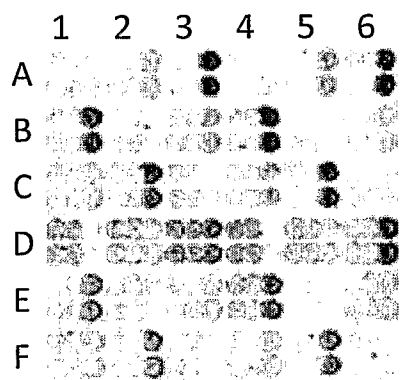
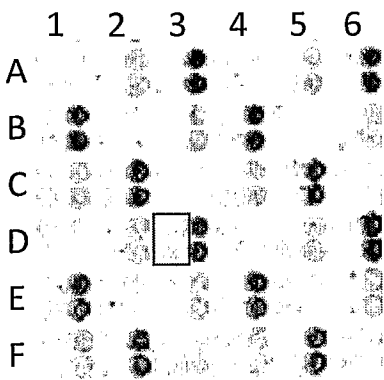


Figure 12

13/60



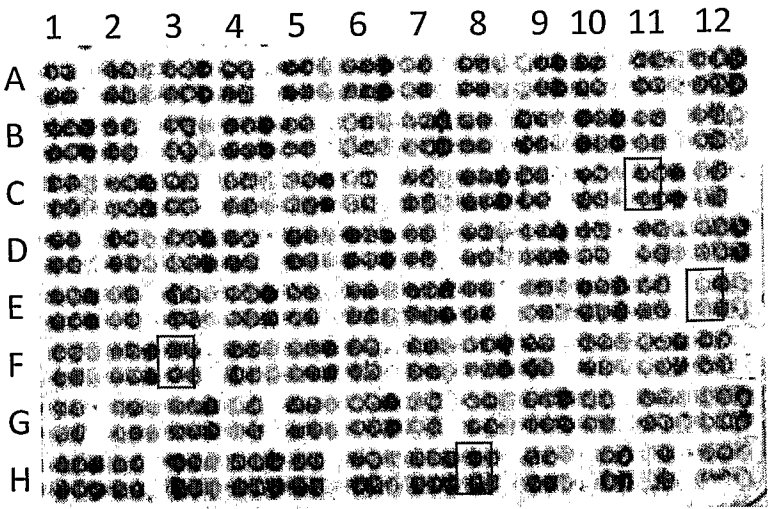
HIV1_1.2
(SEQ ID NO:320)



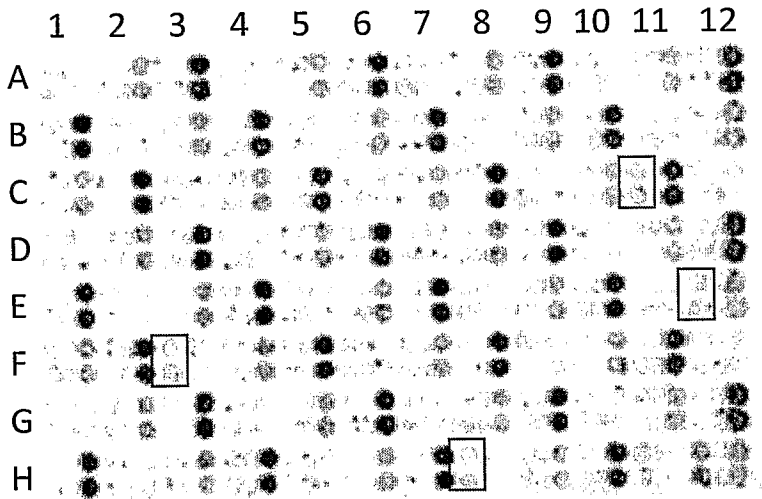
HIV1_1
(SEQ ID NO:319)

Figure 13

14/60



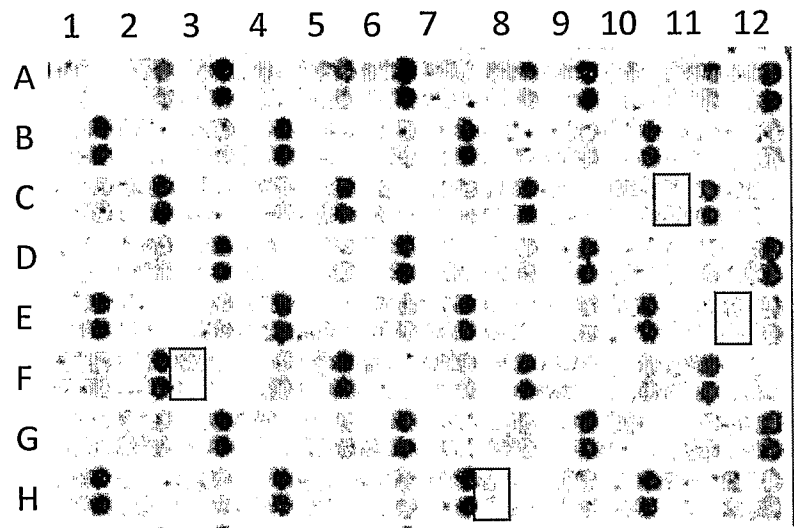
HIV1_1.3
(SEQ ID NO:321)



HIV1_1.5
(SEQ ID NO:323)

Figure 14

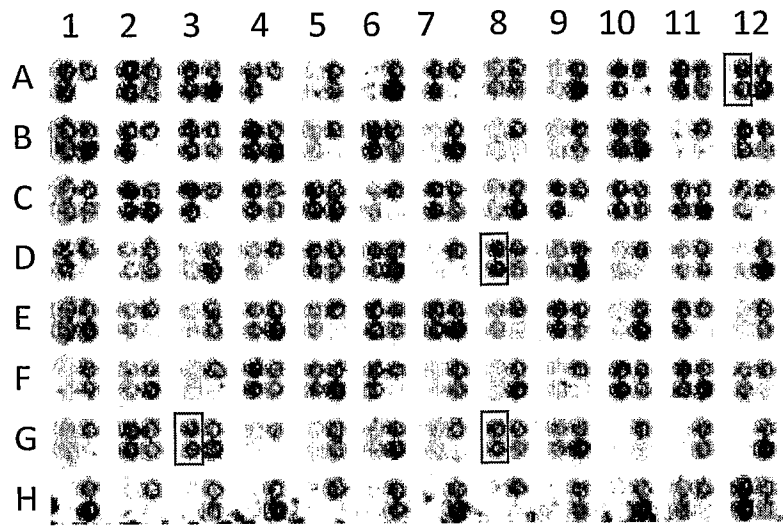
15/60



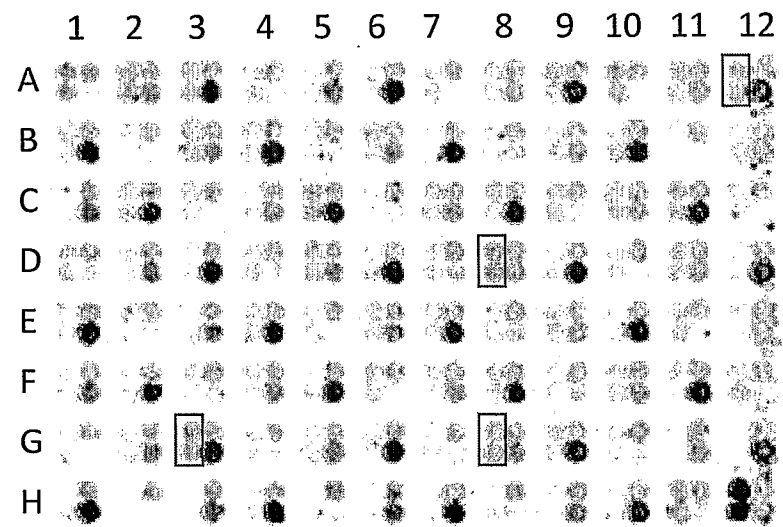
HIV1_1
(SEQ ID NO:319)

Figure 15

16/60



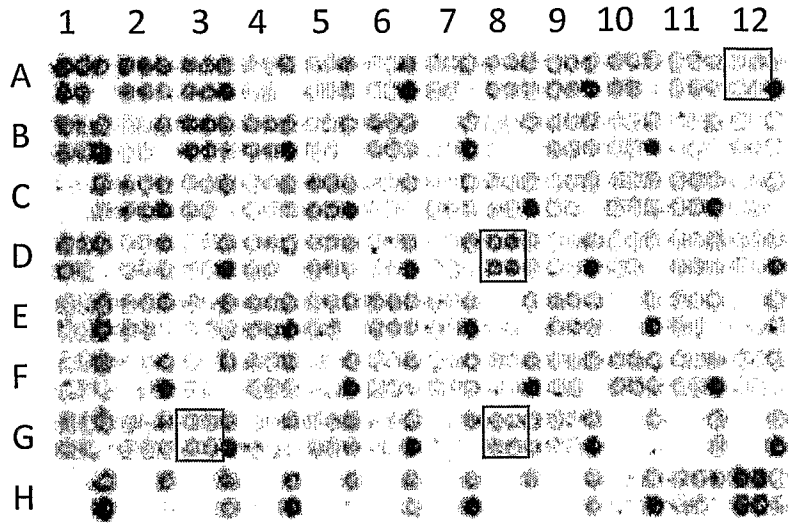
HIV1_1.3
(SEQ ID NO:321)



HIV1_1.5
(SEQ ID NO:323)

Figure 16

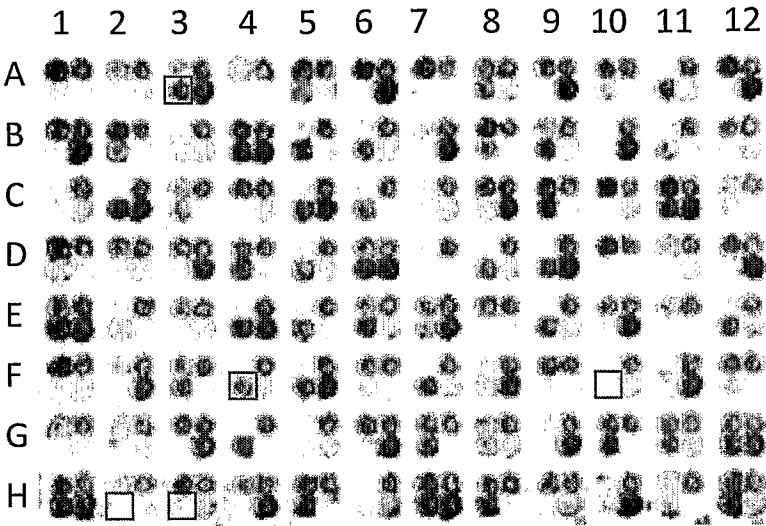
17/60



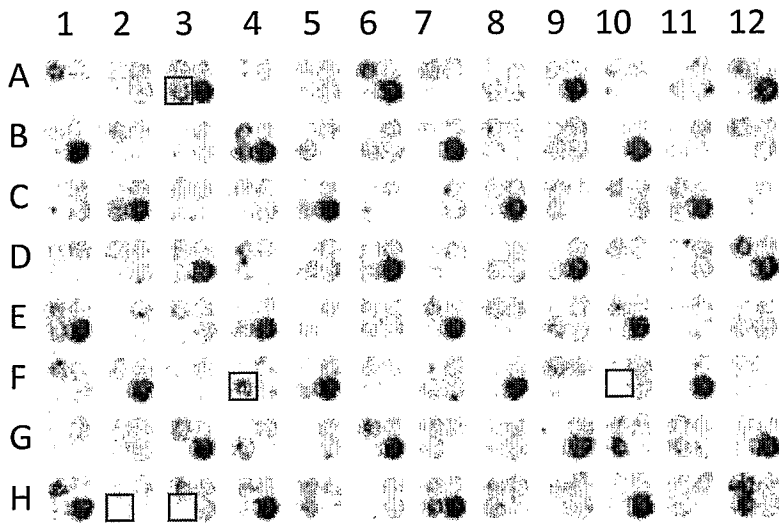
HIV1_1
(SEQ ID NO:319)

Figure 17

18/60



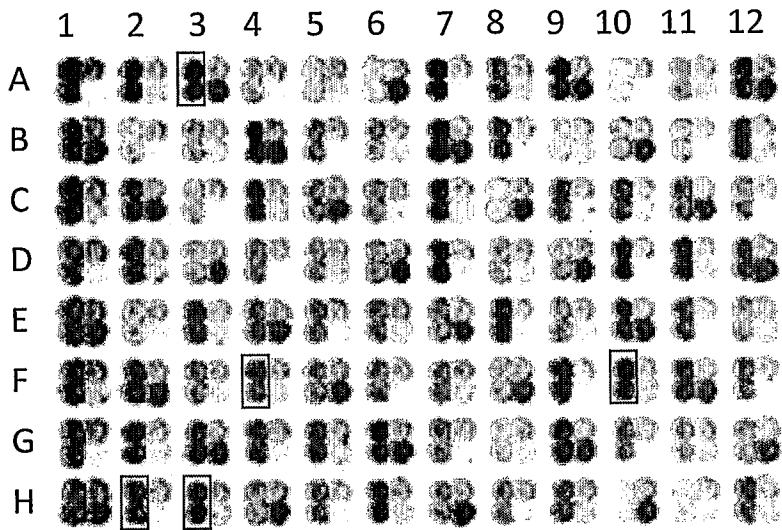
HIV1_1.3
(SEQ ID NO:321)



HIV1_1.5
(SEQ ID NO:323)

Figure 18

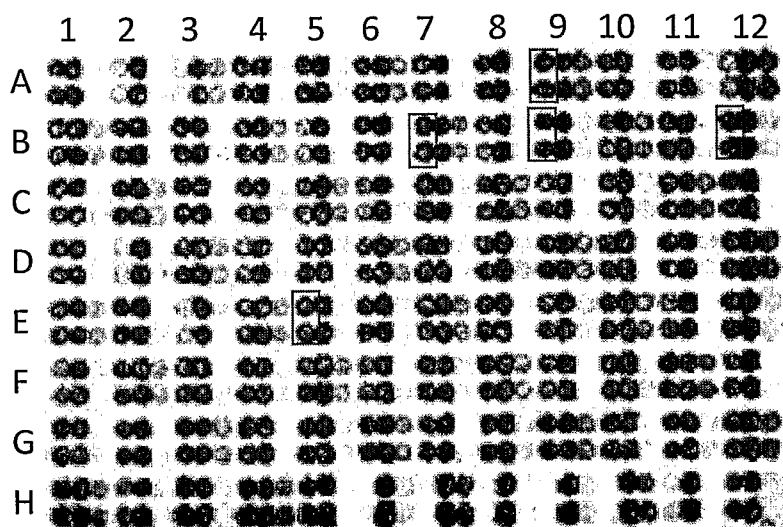
19/60



HIV1_1
(SEQ ID NO:319)

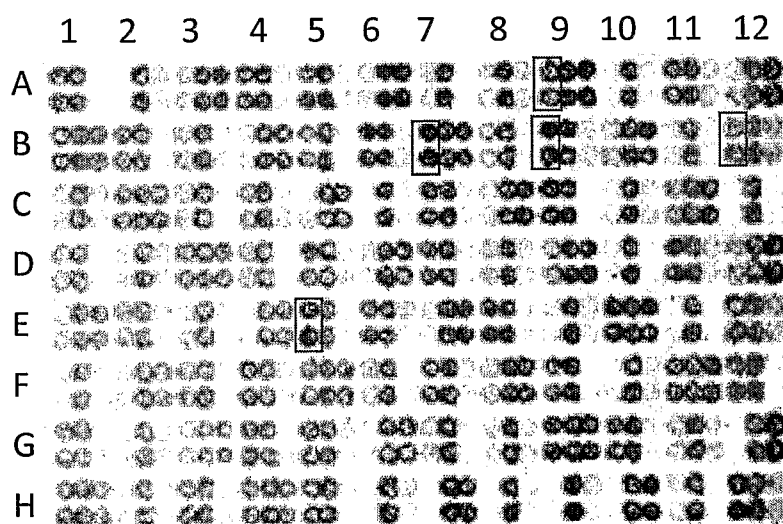
Figure 19

20/60



HIV1_1.4

(SEQ ID NO:322)

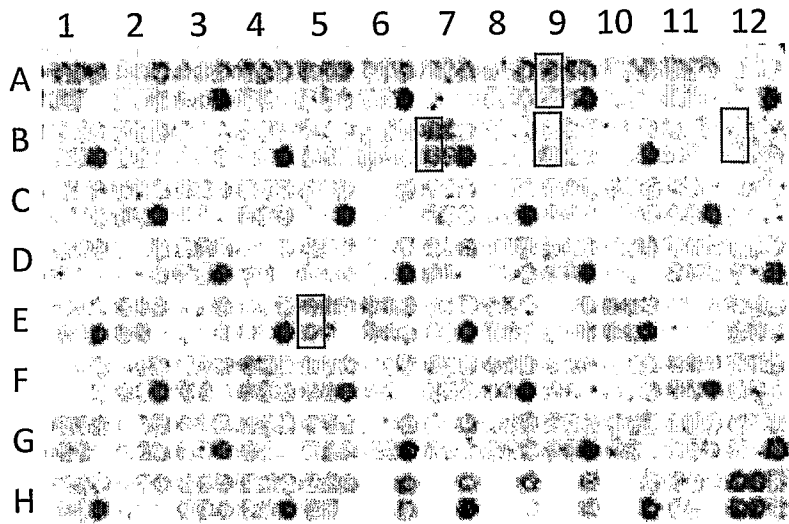


HIV1_1.6

(SEQ ID NO:324)

Figure 20

21/60

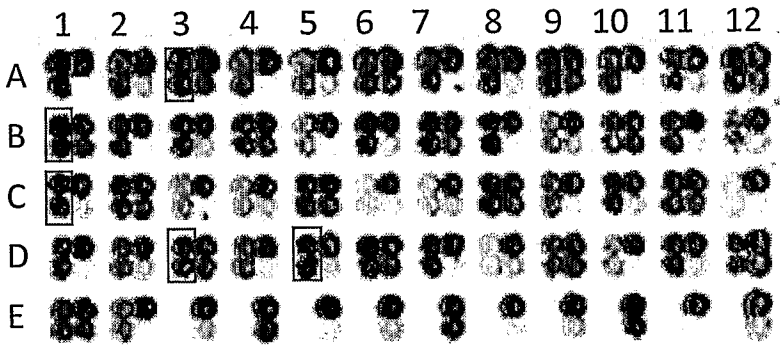


HIV1_1

(SEQ ID NO:319)

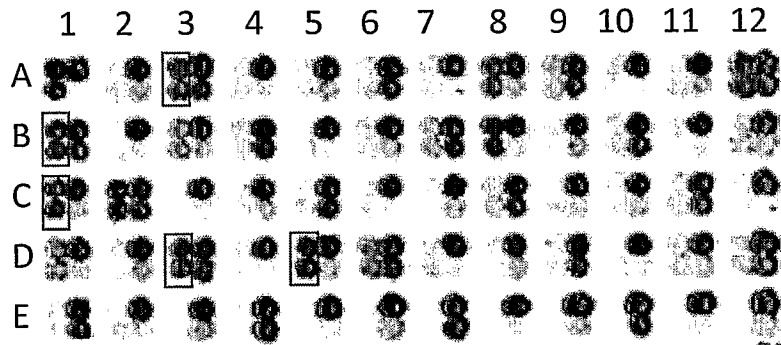
Figure 21

22/60



HIV1_1.4

(SEQ ID NO:322)



HIV1_1.6

(SEQ ID NO:324)

Figure 22

23/60

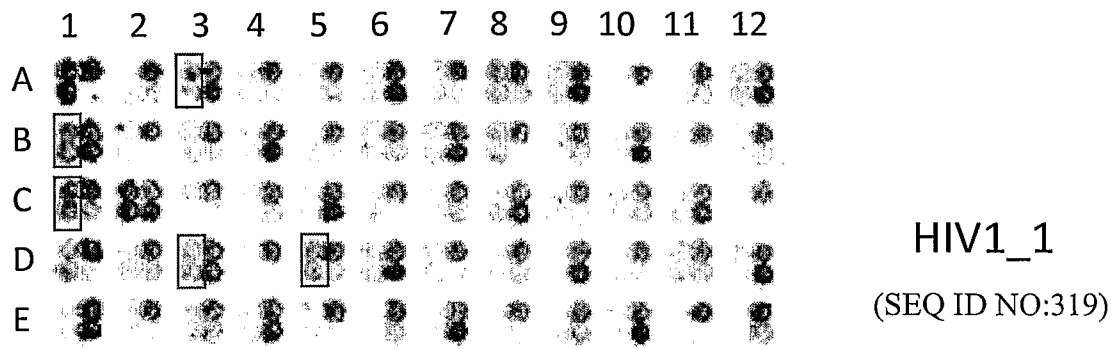


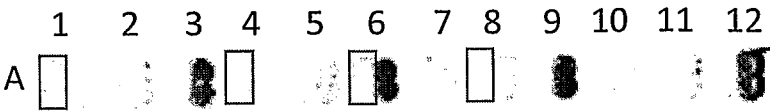
Figure 23

24/60

		⁻¹⁰		⁻⁵	⁻²	⁺²	⁺⁵		⁺¹⁰		
HIV1_3	T	CAG	AC	CCT	TTTA		GTC	AG	TGT	G	(SEQ ID NO:325)
HIV1_3.2	T	CAG	AC	CCT	GTAC		GTC	AG	TGT	G	(SEQ ID NO:326)
HIV1_3.3	T	CAG	AC	CCT	GTAC		AGG	GT	CTG	A	(SEQ ID NO:327)
HIV1_3.4	C	ACA	CT	GAC	GTAC		GTC	AG	TGT	G	(SEQ ID NO:328)
HIV1_3.5	T	CAG	AC	CCT	TTTA		AGG	GT	CTG	A	(SEQ ID NO:329)
HIV1_3.6	C	ACA	CT	GAC	TTTA		GTC	AG	TGT	G	(SEQ ID NO:330)

Figure 24

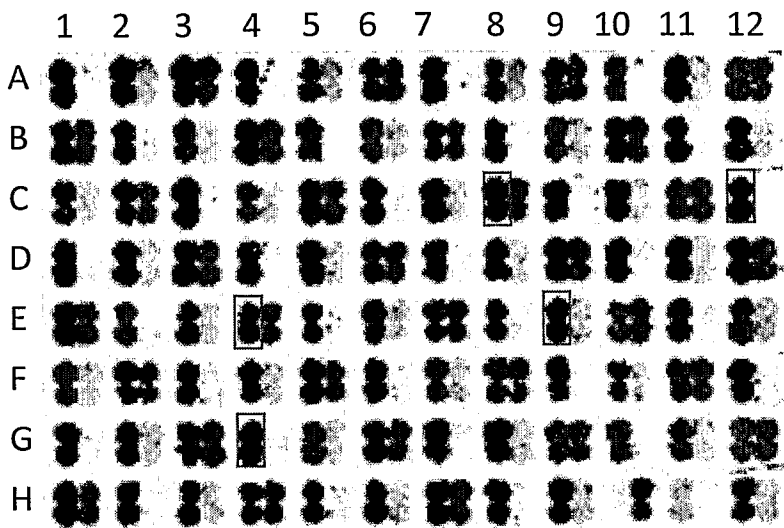
25/60



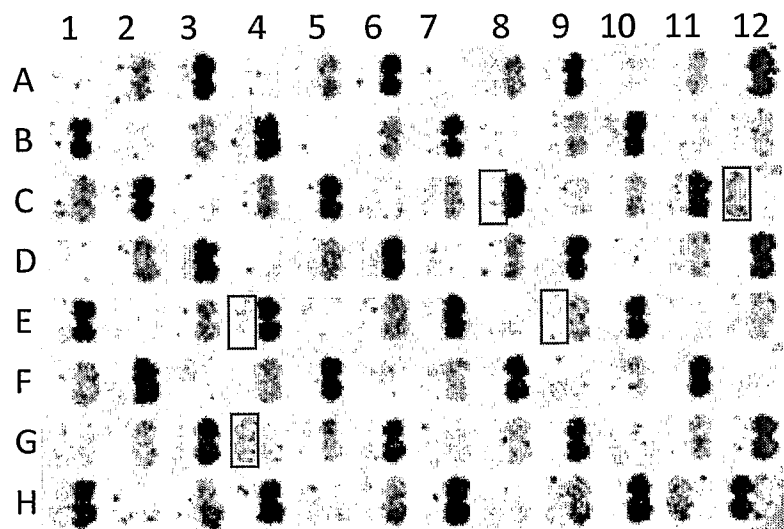
HIV1_3.3
(SEQ ID NO:327)

Figure 25

26/60



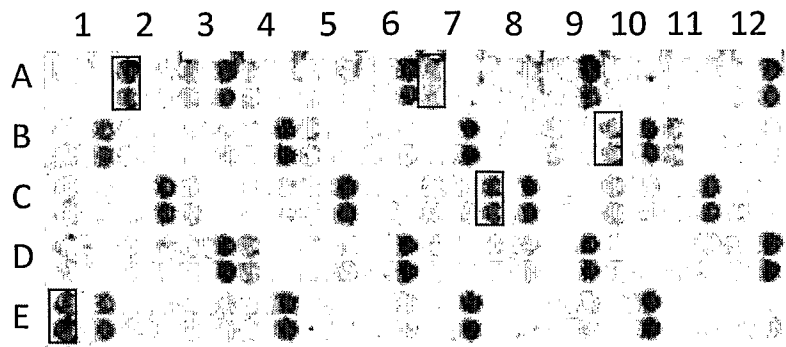
HIV1_3.4
(SEQ ID NO:328)



HIV1_3.6
(SEQ ID NO:330)

Figure 26

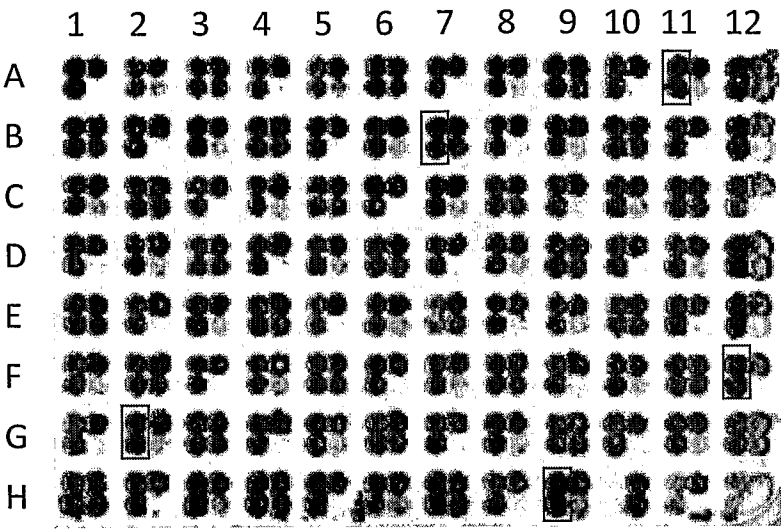
27/60



HIV1_3.3
(SEQ ID NO:327)

Figure 27

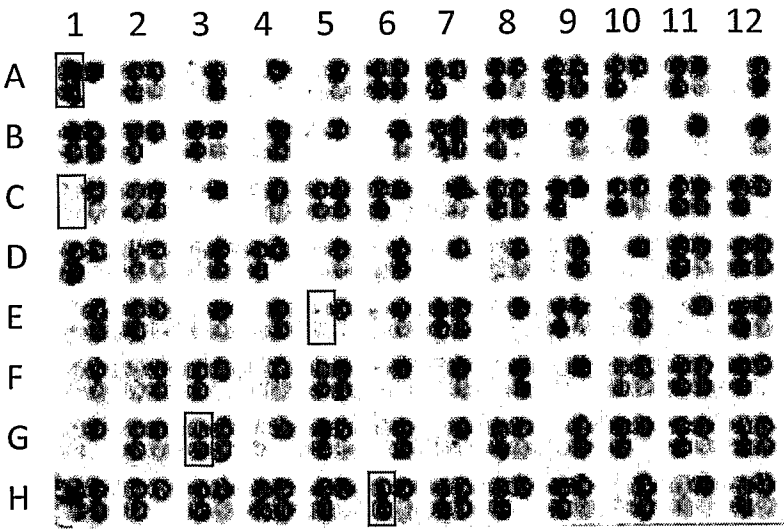
28/60



HIV1_3.3
(SEQ ID NO:327)

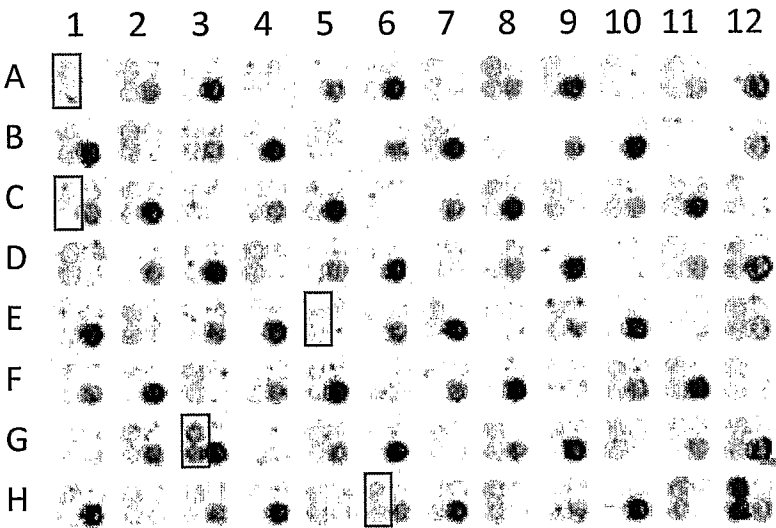
Figure 28

29/60



HIV1_3.3

(SEQ ID NO:327)

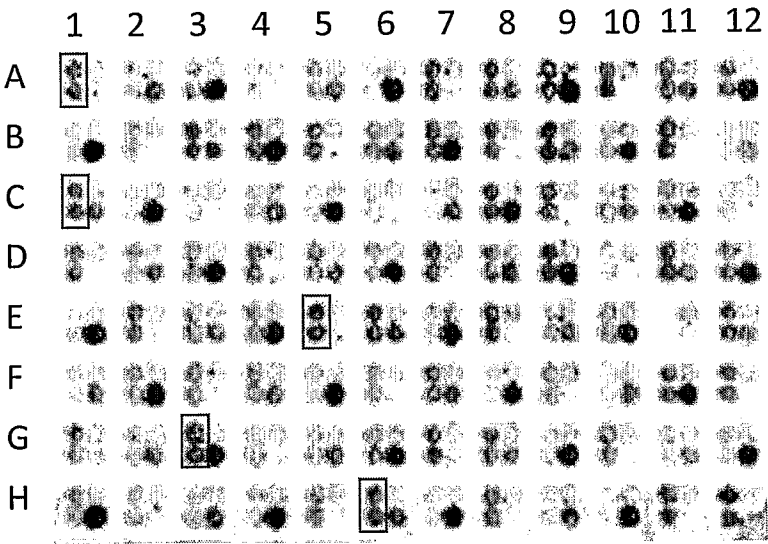


HIV1_3.5

(SEQ ID NO:329)

Figure 29

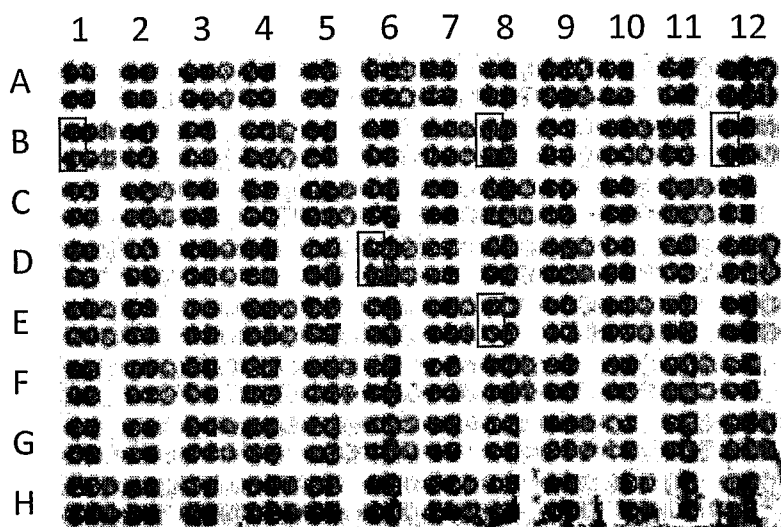
30/60



HIV1_3
(SEQ ID NO:325)

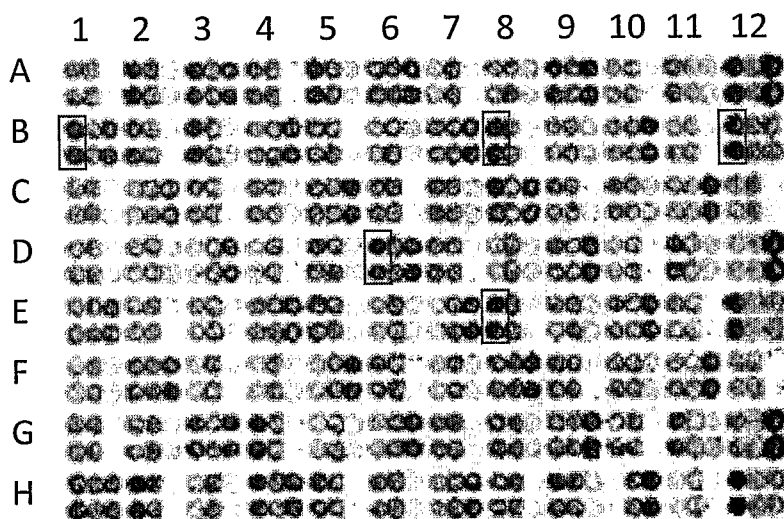
Figure 30

31/60



HIV1_3.4

(SEQ ID NO:328)

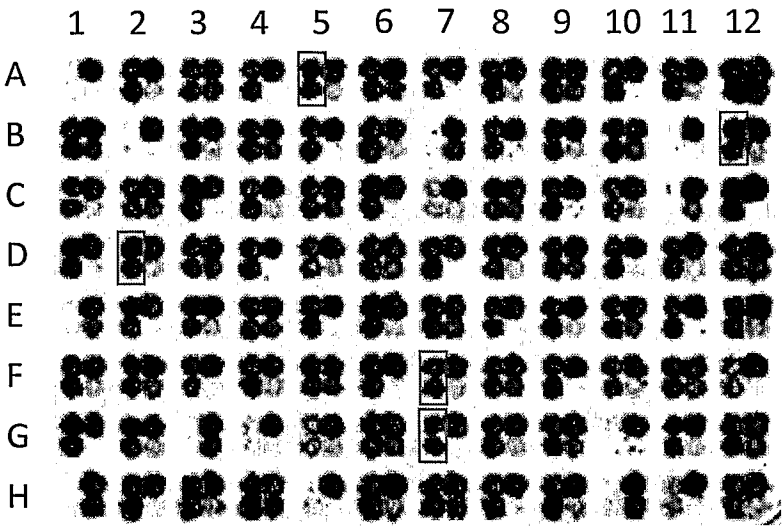


HIV1_3.6

(SEQ ID NO:330)

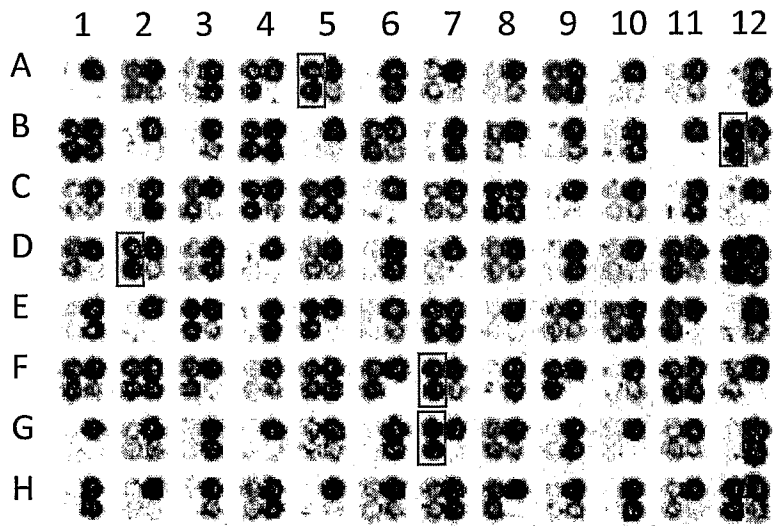
Figure 31

32/60



HIV1_3.4

(SEQ ID NO:328)

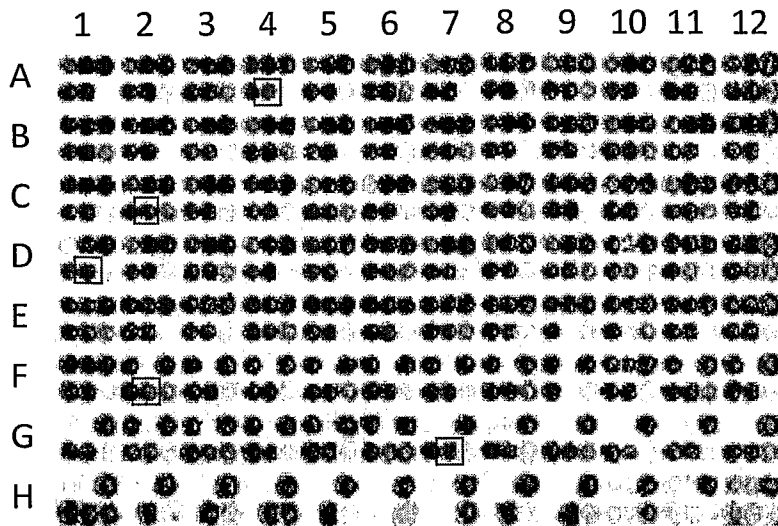


HIV1_3.6

(SEQ ID NO:330)

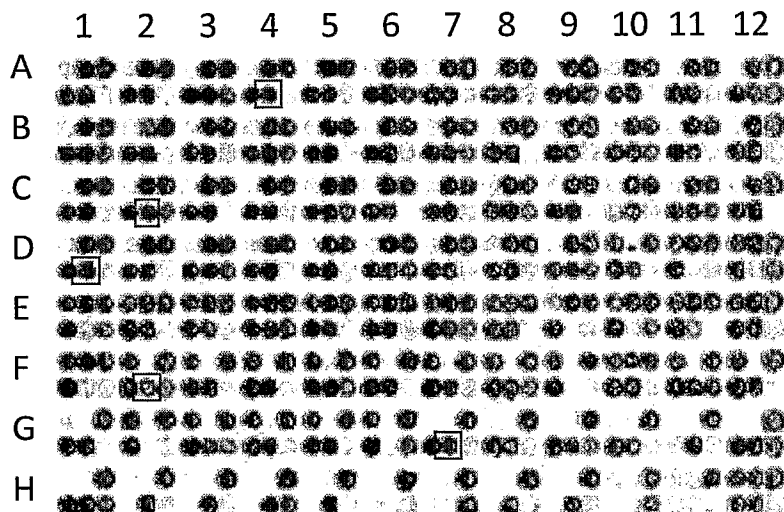
Figure 32

33/60



HIV1_3.4

(SEQ ID NO:328)

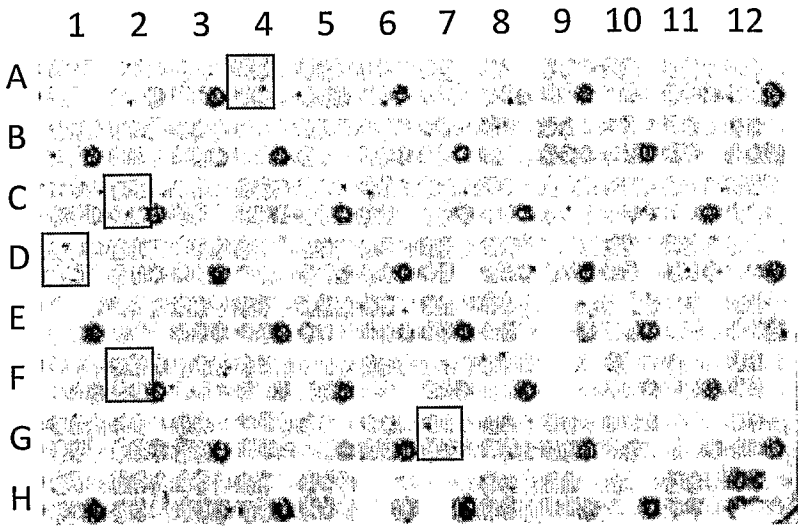


HIV1_3.6

(SEQ ID NO:330)

Figure 33

34/60



HIV1_3
(SEQ ID NO:325)

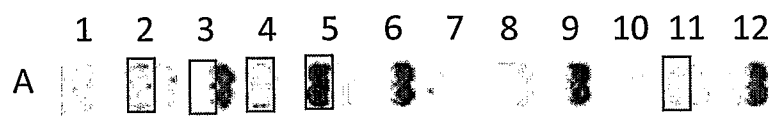
Figure 34

35/60

		⁻¹⁰		⁻⁵	⁻²	⁺²	⁺⁵		⁺¹⁰		
HIV1_4	C	AGC	AT	TCT	GGAC	ATA	AG	ACA	A	(SEQ ID NO:331)	
HIV1_4.2	C	AGC	AT	TCT	GTAC	ATA	AG	ACA	A	(SEQ ID NO:332)	
HIV1_4.3	C	AGC	AT	TCT	GTAC	AGA	AT	GCT	G	(SEQ ID NO:333)	
HIV1_4.4	T	TGT	CT	TAT	GTAC	ATA	AG	ACA	A	(SEQ ID NO:334)	
HIV1_4.5	C	AGC	AT	TCT	GGAC	AGA	AT	GCT	G	(SEQ ID NO:335)	
HIV1_4.6	T	TGT	CT	TAT	GGAC	ATA	AG	ACA	A	(SEQ ID NO:336)	

Figure 35

36/60

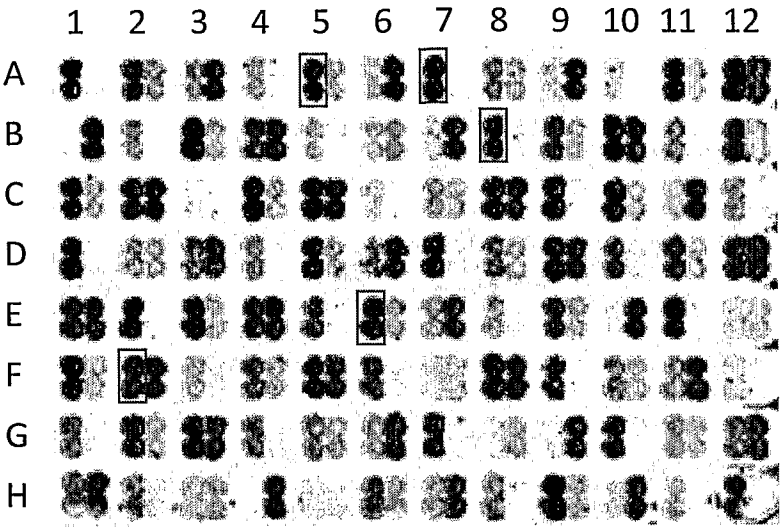


HIV1_4.3

(SEQ ID NO:333)

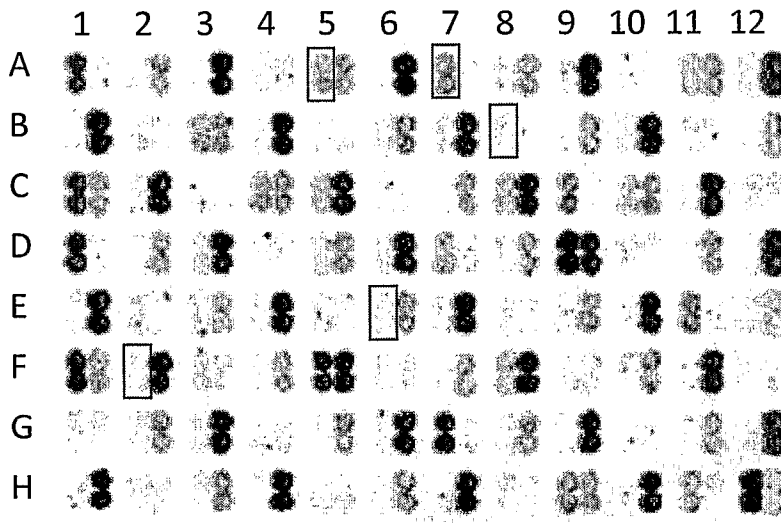
Figure 36

37/60



HIV1_4.4

(SEQ ID NO:334)

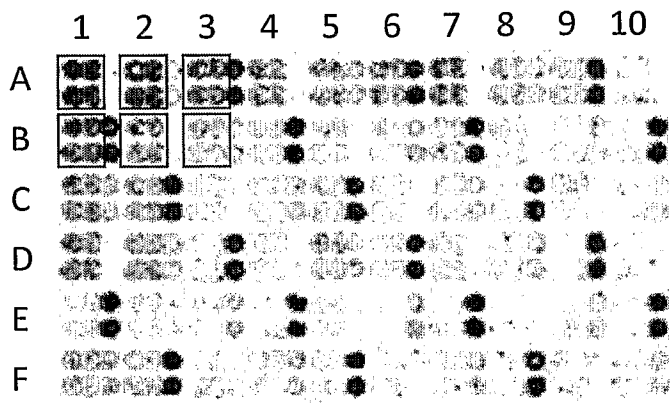


HIV1_4.6

(SEQ ID NO:336)

Figure 37

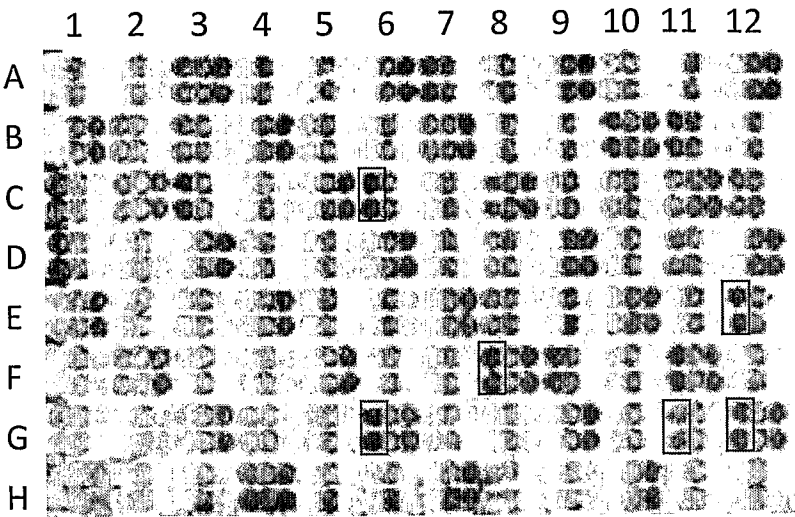
38/60



HIV1_4.2
(SEQ ID NO:332)

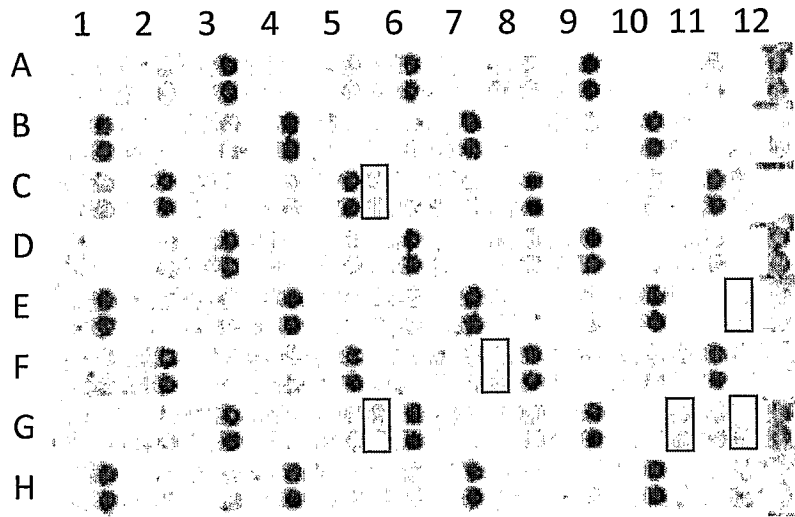
Figure 38

39/60



HIV1_4.3

(SEQ ID NO:333)

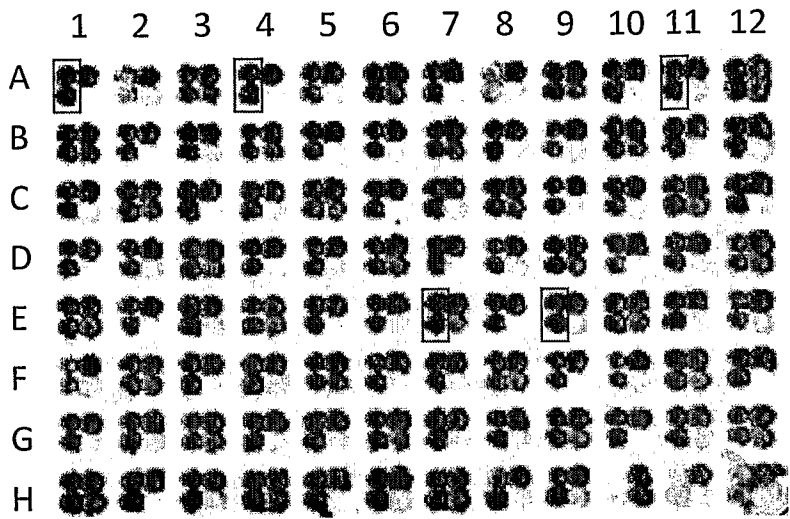


HIV1_4.5

(SEQ ID NO:335)

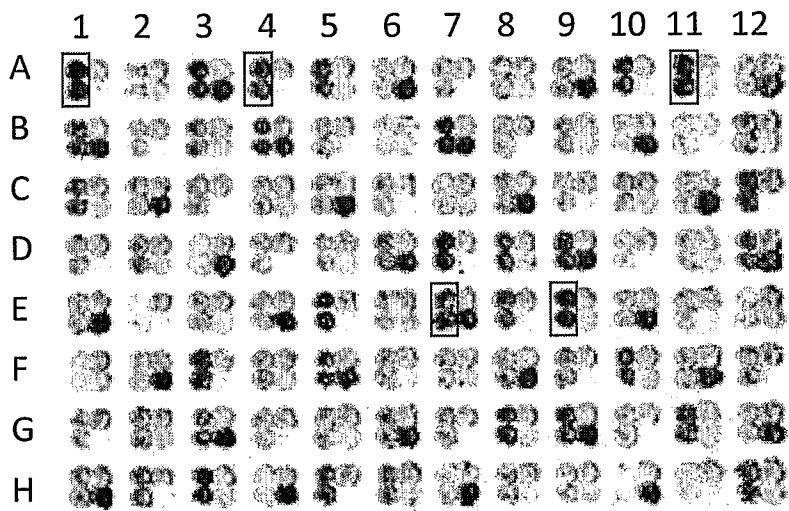
Figure 39

40/60



HIV1_4.3

(SEQ ID NO:333)

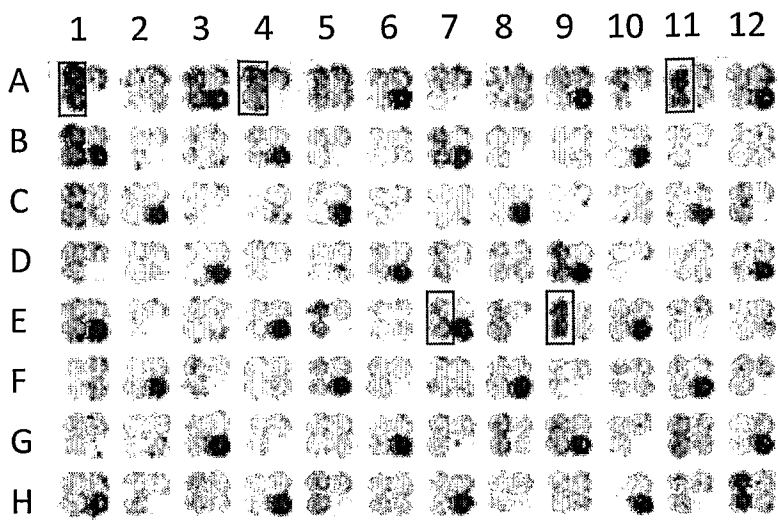


HIV1_4.5

(SEQ ID NO:335)

Figure 40

41/60

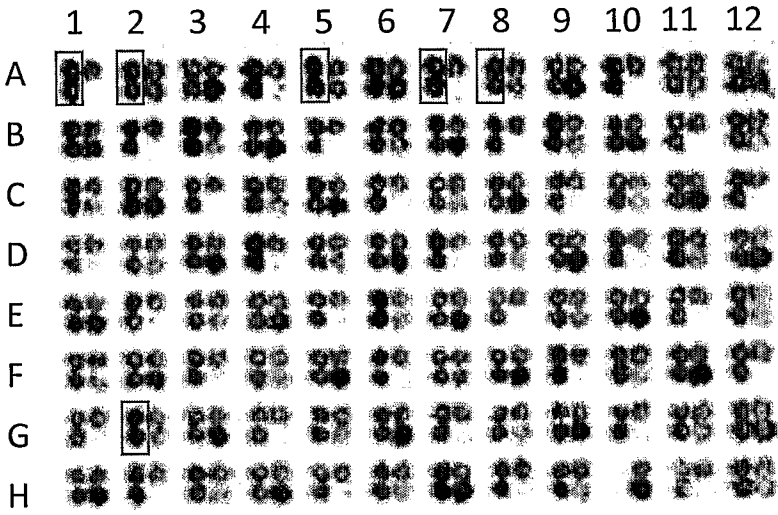


HIV1_4

(SEQ ID NO:331)

Figure 41

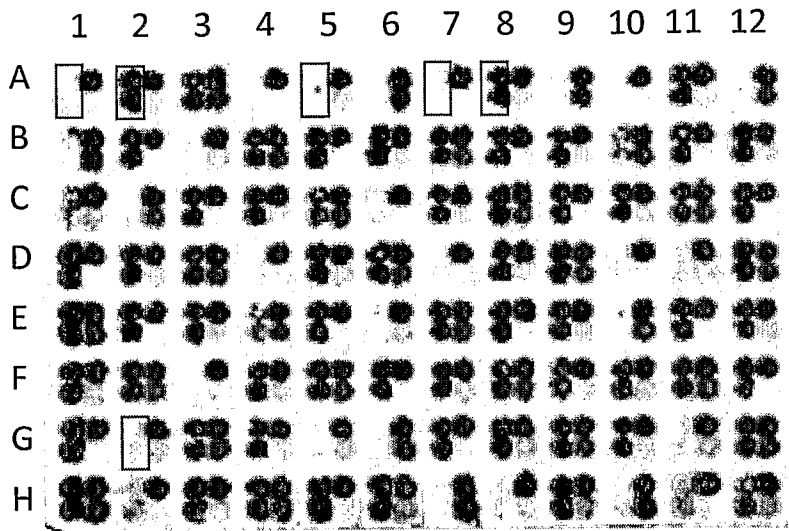
42/60



HIV1_4
(SEQ ID NO:331)

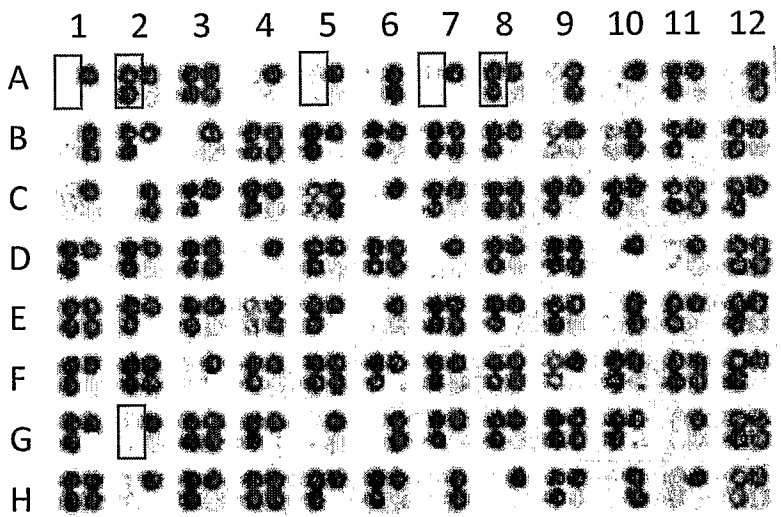
Figure 42

43/60



HIV1_4.3

(SEQ ID NO:333)

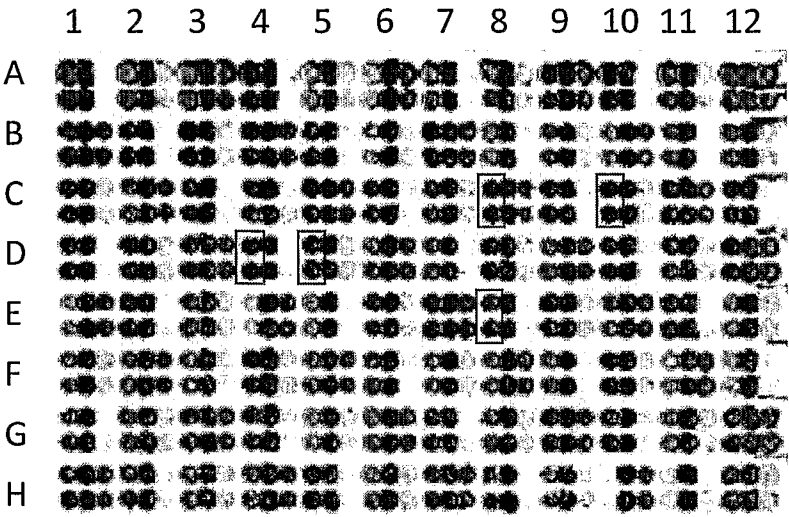


HIV1_4.5

(SEQ ID NO:335)

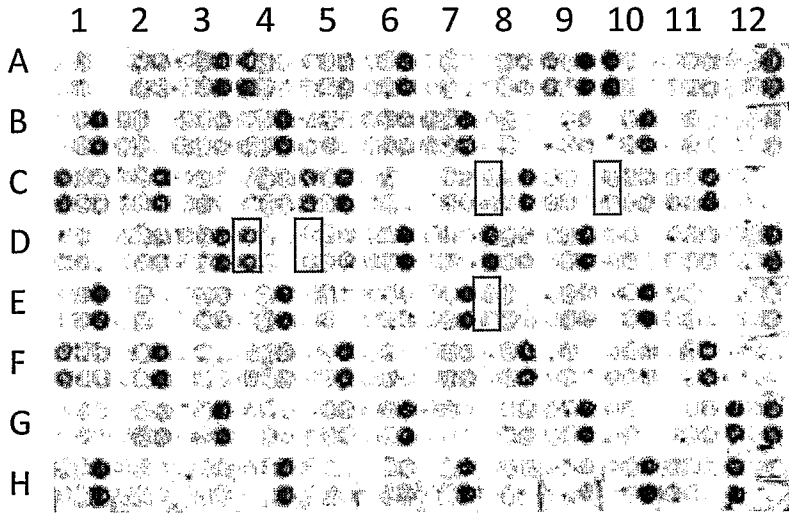
Figure 43

44/60



HIV1_4.4

(SEQ ID NO:334)

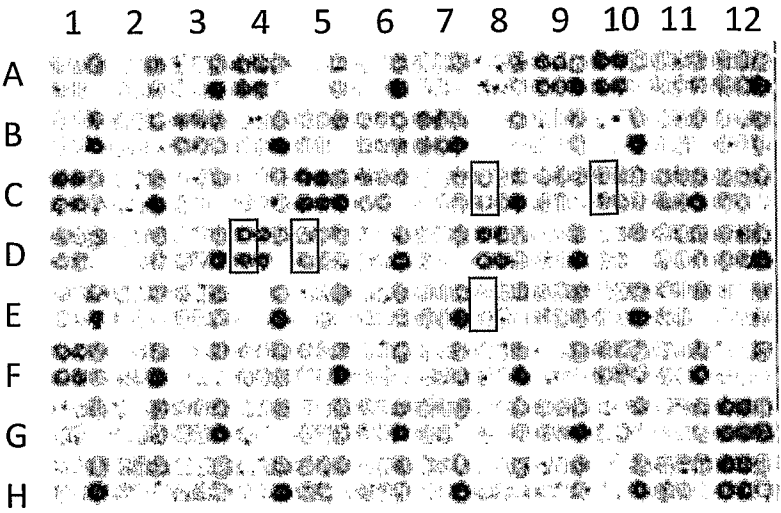


HIV1_4.6

(SEQ ID NO:336)

Figure 44

45/60

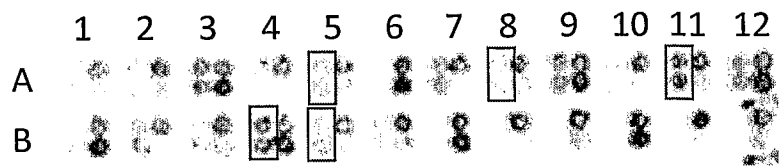


HIV1_4

(SEQ ID NO:331)

Figure 45

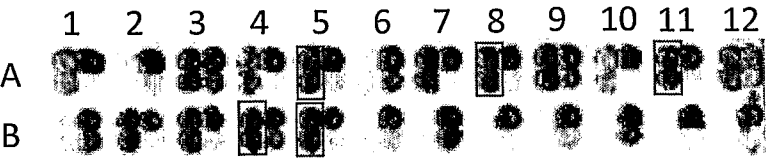
46/60



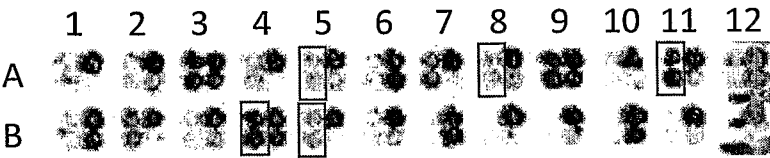
HIV1_4
(SEQ ID NO:331)

Figure 46

47/60



HIV1_4.4
(SEQ ID NO:334)



HIV1_4.6
(SEQ ID NO:336)

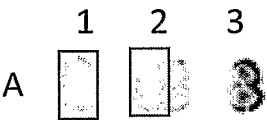
Figure 47

48/60

		⁻¹⁰		⁻⁵	⁻²	⁺²	⁺⁵		⁺¹⁰		
HIV1_5	C	TCT	AT	TAG	ATAC	AGG	AG	CAG	A	(SEQ ID NO:337)	
HIV1_5.2	C	TCT	AT	TAG	GTAC	AGG	AG	CAG	A	(SEQ ID NO:338)	
HIV1_5.3	C	TCT	AT	TAG	GTAC	CTA	AT	AGA	G	(SEQ ID NO:339)	
HIV1_5.4	T	CTG	CT	CCT	GTAC	AGG	AG	CAG	A	(SEQ ID NO:340)	
HIV1_5.5	C	TCT	AT	TAG	ATAC	CTA	AT	AGA	G	(SEQ ID NO:341)	
HIV1_5.6	T	CTG	CTC	CT	ATAC	AGG	AG	CAG	A	(SEQ ID NO:342)	

Figure 48

49/60

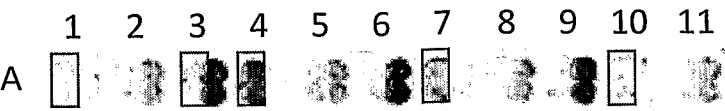


HIV1_5.3

(SEQ ID NO:339)

Figure 49

50/60



HIV1_5.4

(SEQ ID NO:340)

Figure 50

51/60

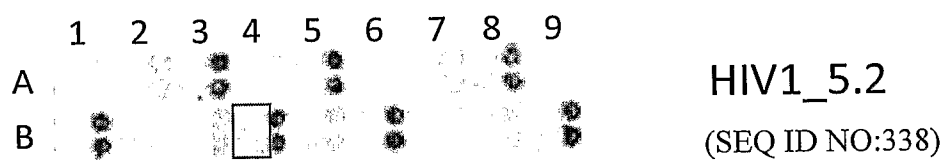


Figure 51

52/60

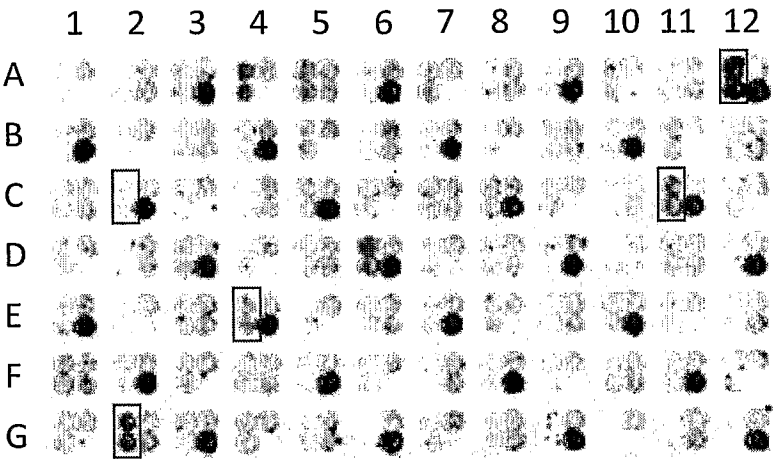


HIV1_5.3

(SEQ ID NO:339)

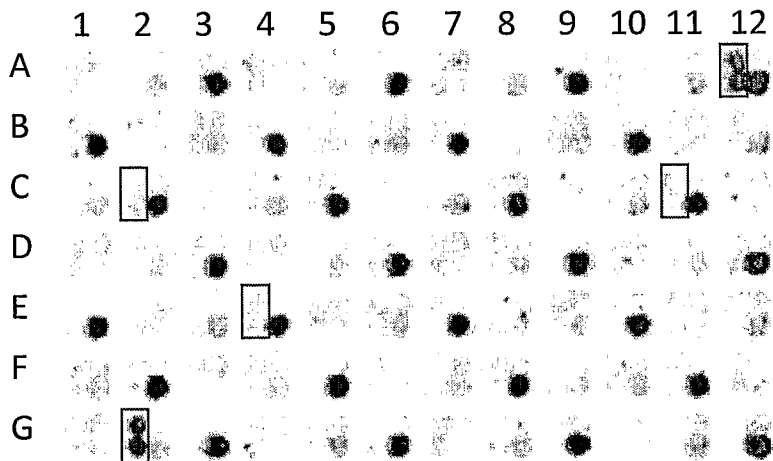
Figure 52

53/60



HIV1_5.3

(SEQ ID NO:339)

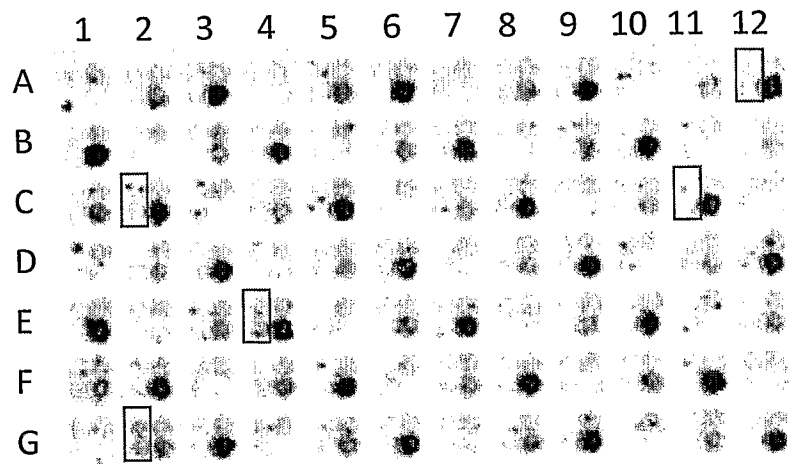


HIV1_5.5

(SEQ ID NO:341)

Figure 53

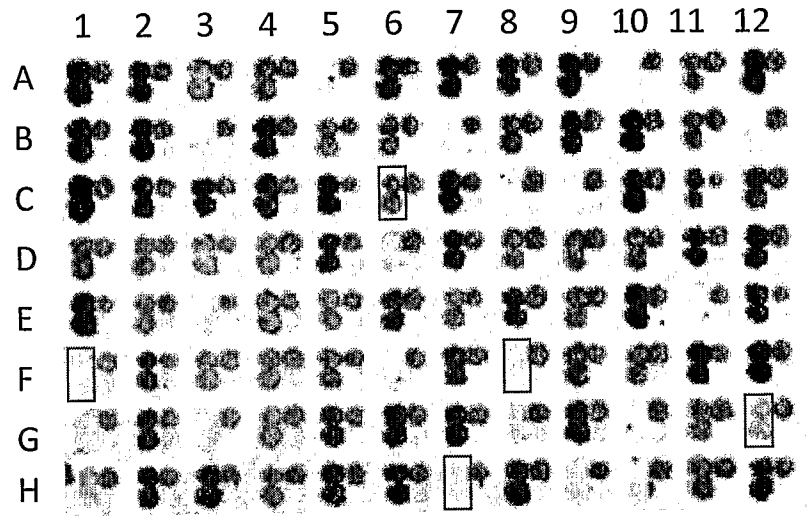
54/60



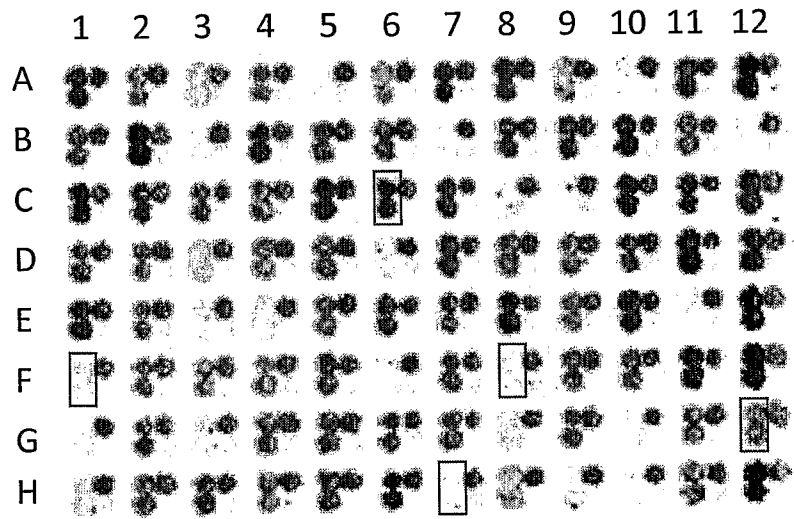
HIV1_5
(SEQ ID NO:337)

Figure 54

55/60



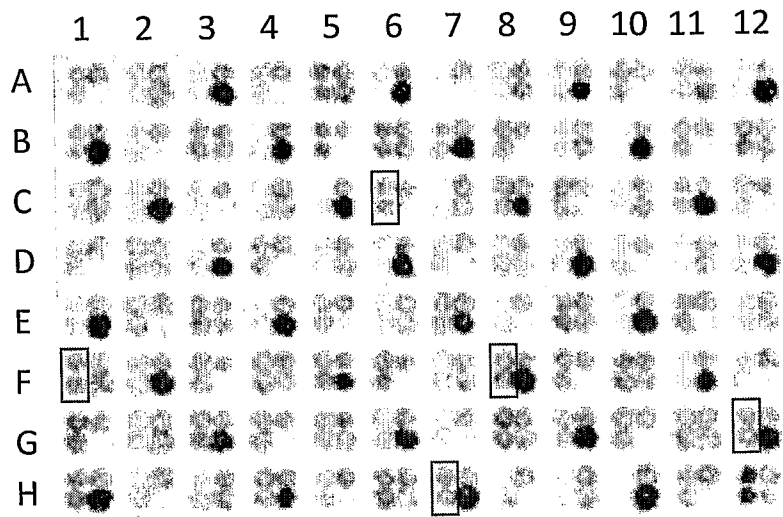
HIV1_5.3
(SEQ ID NO:339)



HIV1_5.5
(SEQ ID NO:341)

Figure 55

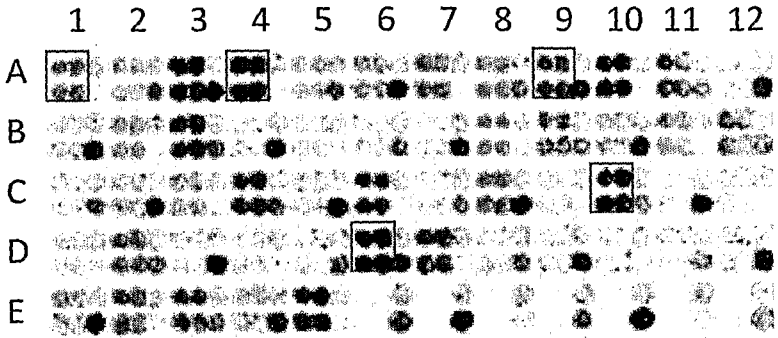
56/60



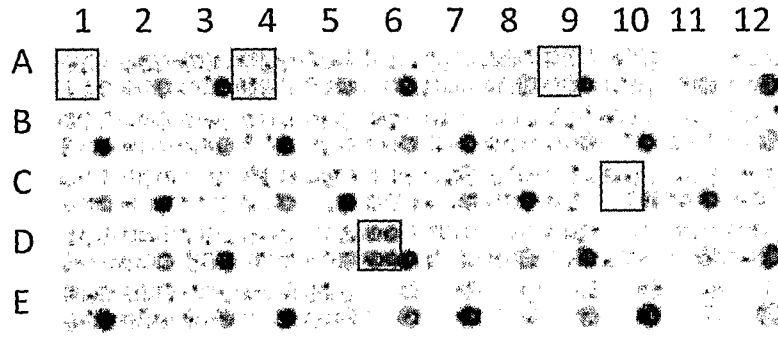
HIV1_5
(SEQ ID NO:337)

Figure 56

57/60



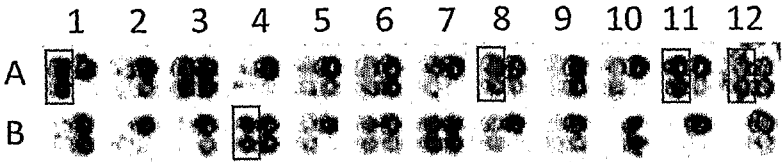
HIV1_5.4
(SEQ ID NO:340)



HIV1_5.6
(SEQ ID NO:342)

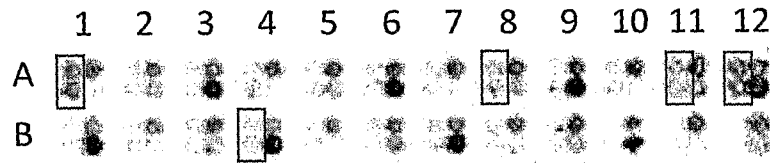
Figure 57

58/60



HIV1_5.4

(SEQ ID NO:340)

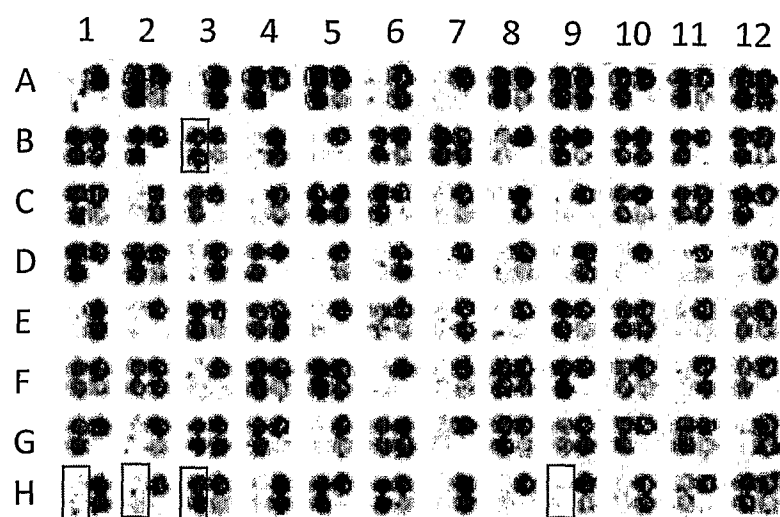


HIV1_5.6

(SEQ ID NO:342)

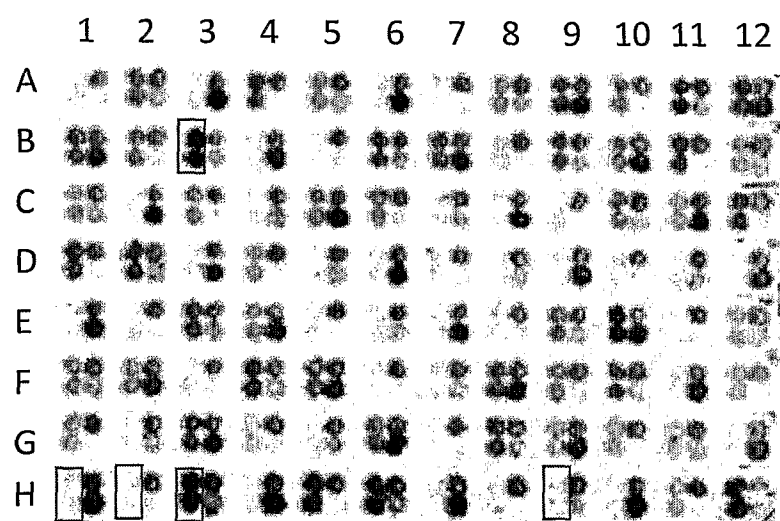
Figure 58

59/60



HIV1_5.4

(SEQ ID NO:340)

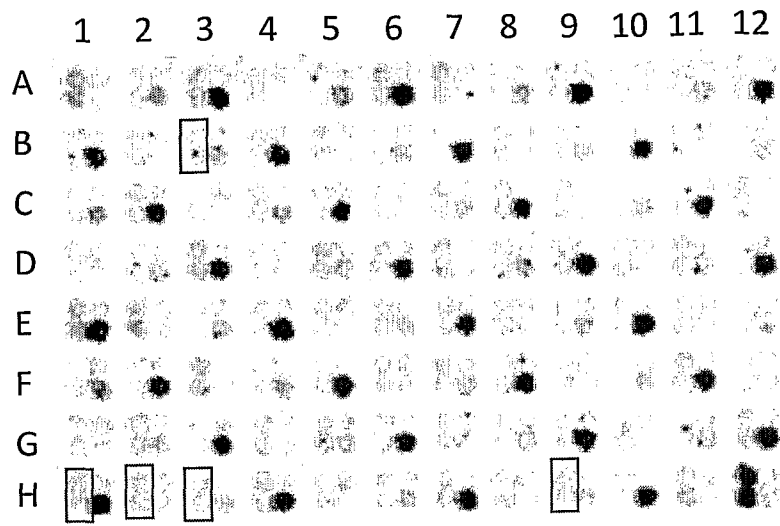


HIV1_5.6

(SEQ ID NO:342)

Figure 59

60/60



HIV1_5
(SEQ ID NO:337)

Figure 60