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(71) **Demandeurs/Applicants:**
JANSSEN VACCINES & PREVENTION B.V., NL;
BETH ISRAEL DEACONESS MEDICAL CENTER, INC.,
US
(72) **Inventeurs/Inventors:**
BAROUCH, DAN H., US;
ABBINK, PETER, US
(74) **Agent:** BORDEN LADNER GERVAIS LLP

(54) **Titre : VECTEURS ADENOVIAUX RECOMBINES SE REPLIQUANT, COMPOSITIONS ET PROCEDES POUR LES UTILISER**
(54) **Title: REPLICATING RECOMBINANT ADENOVIRUS VECTORS, COMPOSITIONS, AND METHODS OF USE THEREOF**

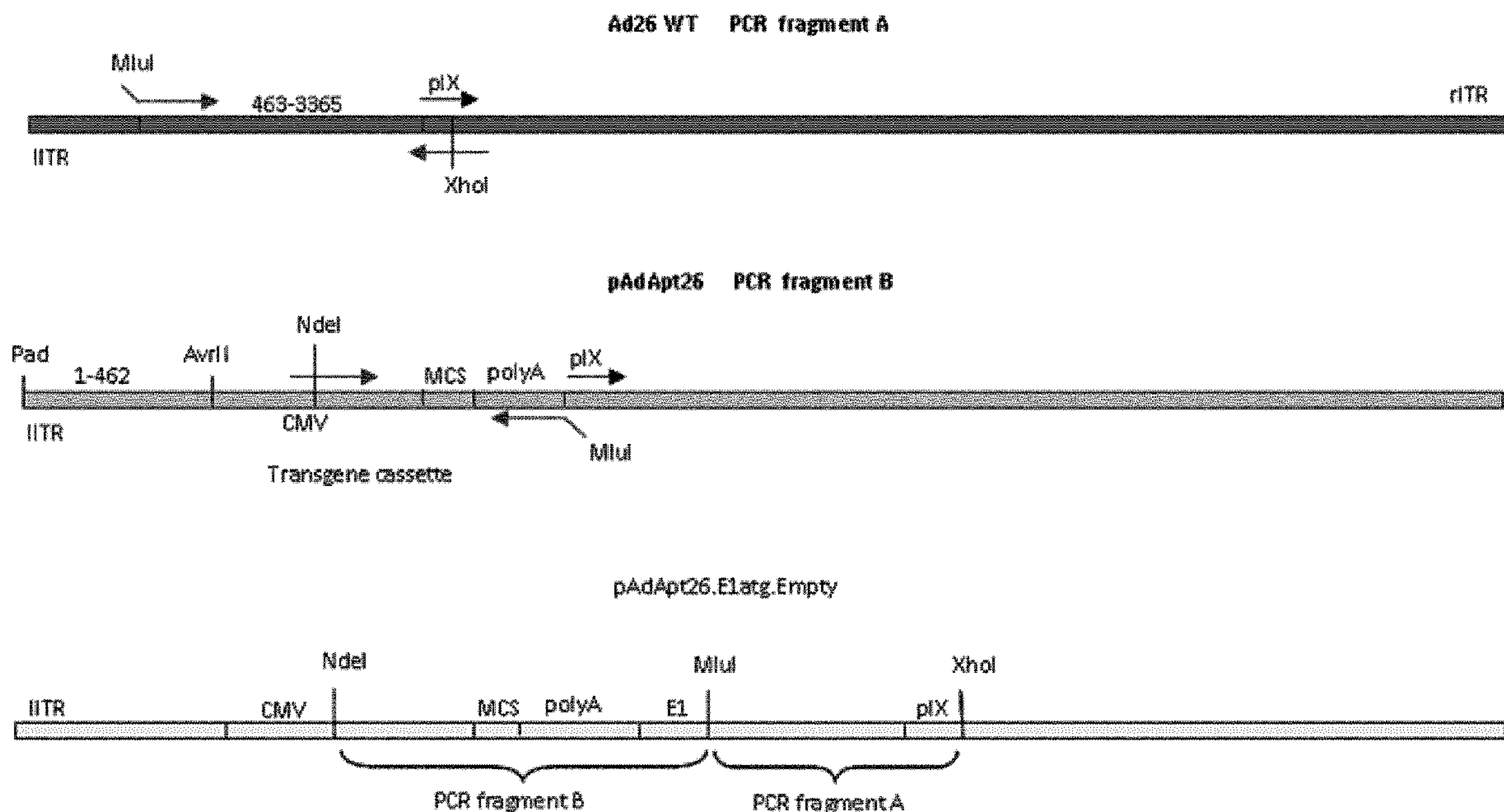


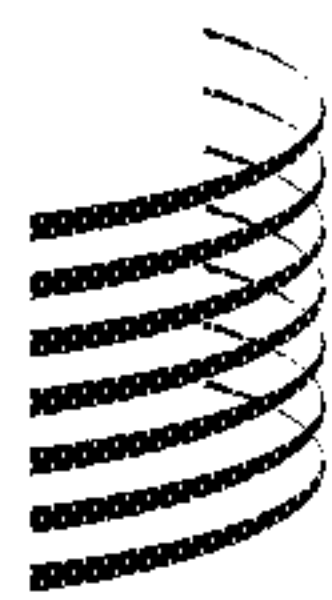
Fig. 2

(57) **Abrégé/Abstract:**

Replicating recombinant adenovirus vectors derived from human adenovirus serotype 26 or human adenovirus serotype 35 are described. The replicating recombinant adenovirus vectors have attenuated replicative capacity as compared to that of the corresponding wild-type adenovirus. They can be used for stable expression of heterologous genes in vivo. Also described are compositions and methods of using these recombinant adenovirus vectors to induce an immune response in a subject, and vaccinate a subject against an immunogenic human immunodeficiency virus (HIV) infection.



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(71) Applicants: **CRUCCELL HOLLAND B.V.** [NL/NL];
Archimedesweg 4, NL-2333 CN Leiden (NL). **BETH IS-
RAEL DEACONESS MEDICAL CENTER INC.**
[—/US]; 330 Brookline Avenue, Brookline, Massachusetts
02215 (US).(72) Inventors: **BAROUCH, Dan H.**; 115 Willard Street, New-
ton, Massachusetts 02461 (US). **ABBINK, Peter**; 150
Hermon St, #3, Winthrop, Massachusetts 02152 (US).(74) Agents: **BESLIER, VICTOR** et al.; P.O. Box 2048,
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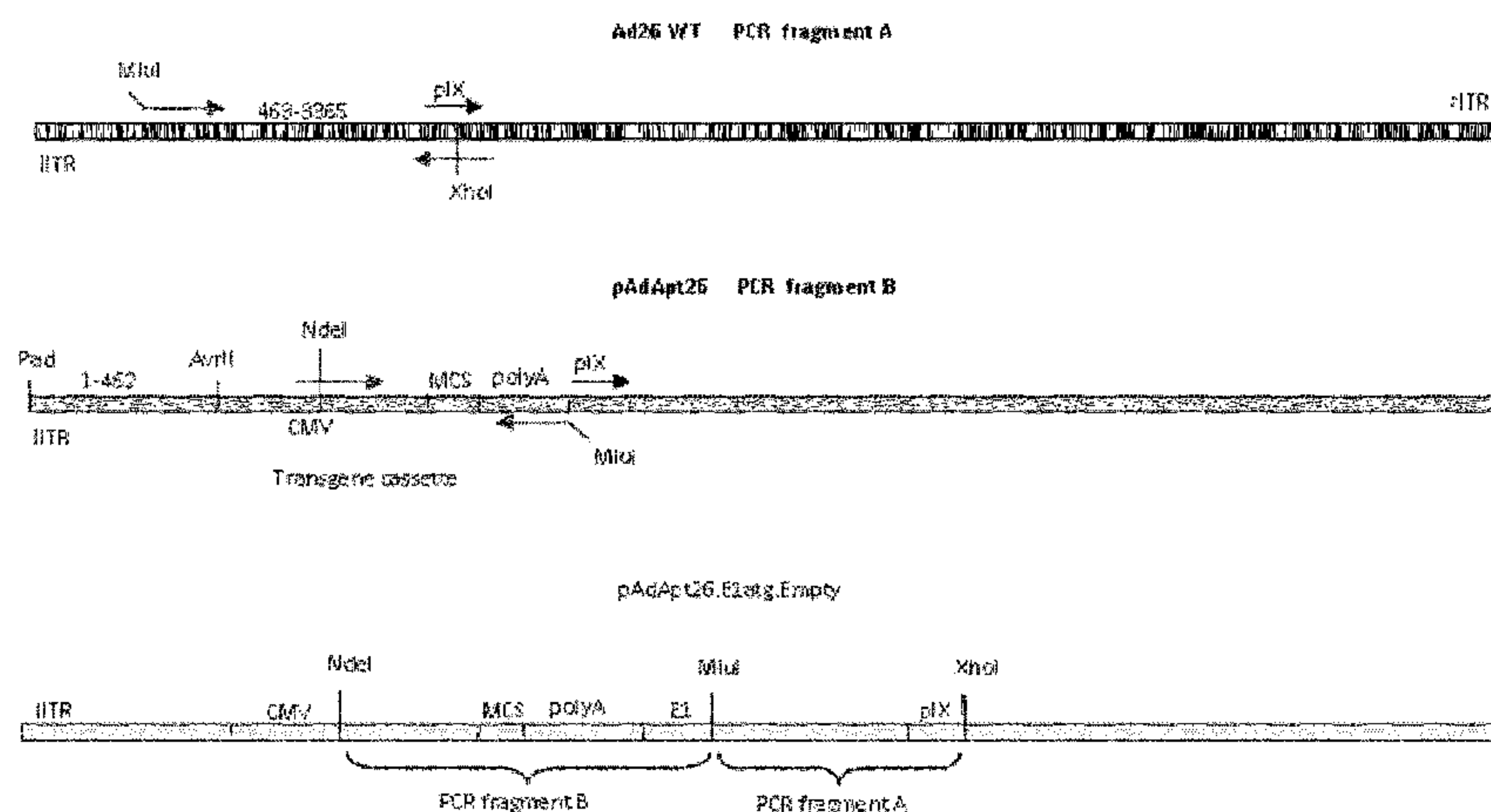
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Fig. 2

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type 35 are described. The replicating recombinant adenovirus vectors have attenuated replicative capacity as compared to that of the
corresponding wild-type adenovirus. They can be used for stable expression of heterologous genes *in vivo*. Also described are com-
positions and methods of using these recombinant adenovirus vectors to induce an immune response in a subject, and vaccinate a
subject against an immunogenic human immunodeficiency virus (HIV) infection.

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TITLE OF THE INVENTION

[0001] Replicating Recombinant Adenovirus Vectors, Compositions, and
Methods of Use Thereof

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CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application is entitled to priority pursuant to 35 U.S.C. § 119(e) to
U.S. Provisional Patent Application No. 61/946,091, filed February 28, 2014, which is
incorporated by reference herein in its entirety.

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FIELD OF THE INVENTION

[0003] The invention relates to replicating recombinant adenovirus vectors. In
particular, the invention provides replicating recombinant adenovirus vectors derived
from human adenovirus serotype 26 (Ad26) or serotype 35 (Ad35) that can be used to
15 induce immune response or provide protective immunity against an HIV infection.

BACKGROUND OF THE INVENTION

[0004] Human Immunodeficiency Virus (HIV) affects millions of people
worldwide, and the prevention of HIV remains a very high priority, even in an era of
20 widespread antiretroviral treatment. In the United States, the Center for Disease
Control (CDC) estimates that of all HIV-positive US residents, approximately one
fifth are unaware of their status, and this small proportion is responsible for
transmitting half the new infections each year [2]. Worldwide, the gap in prompt
diagnosis and treatment is far greater. At the end of 2010, an estimated 34 million
25 people were living with HIV worldwide, up 17% from 2001. Although the majority
of new HIV infections continue to occur in sub-Saharan Africa, the CDC estimated
that the annual incidence of HIV infection from 2008-2011 in the United States has
remained stable at around 15-16/100,000, with over 40,000 new infections each year.
Thus, it is an urgent global health priority to find a safe and potent HIV vaccine that
30 would prevent HIV infection or blunt its initial impact prior to diagnosis, including
both destruction of the gut CD4 pool [3] and high risk of transmission [4].

[0005] Live attenuated vaccines have proven to be highly efficacious in
humans and in non-human primates (NHP) against certain viral diseases, such as a
live attenuated simian immunodeficiency virus (SIV) based vaccine for preventing

SIV infection. Unfortunately, due to safety risks associated with live attenuated HIV, such a strategy is not applicable for HIV human vaccine.

[0006] As an alternative to live attenuated viral vaccines, the use of replication incompetent recombinant viral vectors has been explored for vaccines and other types of gene therapy. In particular, replication incompetent recombinant adenoviral vectors, particularly adenovirus serotypes 2 and 5 (Ad2 and Ad5) have been extensively studied for gene delivery applications, including vaccination. Although such replication incompetent Ad5 vector-based vaccines have been shown to elicit protective immune responses in a variety of animal models, the utility of recombinant Ad5 vector-based vaccines for human immunodeficiency virus (HIV) and other pathogens is likely to be limited by the high seroprevalence of Ad5-specific neutralizing antibodies (NAbs) in human populations [17]. For example, in a seroepidemiology study of 4,381 subjects worldwide, it was observed that Ad5 NAb titers were nearly universal and high titer in sub-Saharan Africa, with the majority of individuals exhibiting Ad5 NAb titers >200 [14].

[0007] Even though Ad5 has high seroprevalence in humans, several HIV-1 vaccine efficacy trials have been conducted using vaccines based on recombinant Ad5 vector-based vaccines. These studies include the HVTN 502 / STEP (Merck Ad5), HVTN 503 / Phambili (Merck Ad5), and HVTN 505 (NIH VRC DNA/Ad5) HIV-1 vaccine efficacy trials. However, all three of these HIV-1 vaccine efficacy studies, which utilized nonreplicating Ad5 and DNA/Ad5 vaccines, showed no efficacy against HIV-1 infection. Moreover, a trend towards increased HIV-1 infection was observed in vaccinees with the Merck Ad5 vaccine from the STEP study as compared with placebos. Experience to date with replication incompetent vectors such as adenovirus subtype 5 for HIV vaccine has been disappointing, with failure to show benefit in several efficacy trials [5-8].

[0008] Accordingly, concerns regarding the safety of Ad5 vectors, particularly from the STEP study [8, 10], have led to the exploration of biologically substantially different Ad vectors from alternative serotypes as viral vaccine vectors [11-13]. One example of an alternative adenovirus serotype to Ad5 is Adenovirus serotype 26 (Ad26). Ad26 is a non-enveloped DNA virus that is a relatively uncommon virus in humans. Ad26 is not known to replicate in any other species. A number of surveys for adenovirus in different populations have shown it to be isolated only rarely, and even when isolated, seldom associated with symptoms. Experimental inoculation,

likewise, showed little evidence for serious infection. See, e.g., [14, 27-43]. Thus, there is no evidence from observational studies that Ad26 causes clinical symptoms in healthy adults, and experimental data from an Ad26 challenge study also suggested that enteric Ad26 infection does not produce symptoms [44].

5 [0009] In terms of at least receptor usage, in vivo tropism, interactions with dendritic cells, innate immune profiles, adaptive immune phenotypes, and protective efficacy against SIV in rhesus monkeys, Ad26 has proven to be biologically very different from Ad5 [11, 12, 15, 19-22]. Moreover, the safety and immunogenicity of nonreplicating Ad26 vector in humans has been demonstrated (ClinicalTrials.Gov
10 NCT01215149). Furthermore, many of the advantageous biological differences between Ad5 and Ad26, such as lower seroprevalance and low neutralizing antibody titers in humans are also present between Ad5 and Ad35.

[0010] Replication-incompetent Ad26 has been tested in a GLP toxicology study and three Phase I clinical trials with no significant pattern of adverse effects.

15 Although replication incompetent viral vectors are preferred for gene therapy and related applications, such as vaccination, since replicating viral vectors can produce multiple copies of the virus, which can go on to infect other cells, setting of an infections cycle, there are some possible drawbacks to the use of replication incompetent viral vectors. One possible drawback of replication-incompetent viral
20 vectors is that expression of the target gene to be delivered to the host from the viral vector can decrease following administration of the vector. Being unable to replicate or propagate in the host, the viral vector cannot produce any new copies that can subsequently be used to augment gene expression, requiring re-administration of the viral vector. If the same adenovirus serotype is re-administered to the host, the host
25 may generate neutralizing antibodies to that particular adenovirus serotype, resulting in a serotype specific anti-adenovirus response. Such a serotype specific anti-adenovirus response may prevent effective re-administration of the viral vector, rendering it less effective as a vaccine or gene delivery vehicle.

[0011] Accordingly, there is a need in the art for new recombinant viral vectors
30 that can be used as vaccine vectors that overcome certain disadvantages associated with replication –incompetent recombinant viral vectors. In particular, there exists a need for new recombinant viral vectors that can be used as vaccine vectors against infectious diseases, such as HIV infection. Such a vaccine preferably would be simple to administer, long-acting, with minimal adverse effects. In the case of an HIV

vaccine, the HIV vaccine further would preferably be effective against a wide scope of the diversity of circulating types of HIV transmission, including the most frequent.

BRIEF SUMMARY OF THE INVENTION

5 [0012] The invention satisfies this need by providing a replicating recombinant adenovirus vector comprising a recombinant adenovirus genome derived from a human adenovirus serotype 26 or serotype 35 genome. In particular, the invention provides a replicating recombinant adenovirus vector that can be used to induce an immune response or provide protective immunity in a subject, e.g. against an HIV
10 infection.

[0013] In one general aspect, the invention provides a replicating recombinant adenovirus vector, comprising a recombinant adenovirus genome having:

- (a) a promoter operably linked to a heterologous nucleic acid sequence;
- (b) a functional E1 coding region;
- 15 (c) a deletion in the E3 coding region; and
- (d) a deletion in the E4 coding region, provided that E4 open reading frame 6/7 is not deleted,

wherein the adenovirus genome is human adenovirus serotype 26 or 35 genome.

20 [0014] According to a preferred embodiment of the invention, the heterologous nucleic acid sequence is located between a left inverted terminal repeat (ITR) and the 5'-end of the functional E1 coding region of the replicating recombinant adenovirus vector.

[0015] In an embodiment of the invention, the replicating recombinant adenovirus
25 vector comprises a heterologous nucleic acid sequence encoding an immunogenic polypeptide. The heterologous nucleic acid sequence can encode an HIV antigen, preferably an HIV antigen derived from the sequences of the HIV *gag*, *pol*, and/or *env* gene products, and more preferably a mosaic HIV antigen. In particular embodiments, the heterologous nucleic acid sequence encodes a polypeptide
30 comprising the amino acid sequence of SEQ ID NO: 48 or SEQ ID NO: 50. In more particular embodiments, the heterologous nucleic acid sequence comprises the nucleotide sequence of SEQ ID NO: 47 or SEQ ID NO: 49.

[0016] In embodiments of the invention, the replicating recombinant adenovirus vector comprises a functional E1 coding region encoding the amino acid sequences of

SEQ ID NOs: 14, 15, and 16. In a particular embodiment, the functional E1 coding region comprises the nucleotide sequence of SEQ ID NO: 13.

[0017] In one embodiment of the invention, the replicating recombinant adenovirus vector comprises a partially deleted E3 coding region, and the partially
5 deleted E3 coding region consists of the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 6. In a particular embodiment, the partially deleted E3 coding region consists of the nucleotide sequence of SEQ ID NO: 5.

[0018] In one embodiment of the invention, the replicating recombinant adenovirus vector comprises a partially deleted E4 coding region, and the partially
10 deleted E4 coding region consists of the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 22. In a particular embodiment, the partially deleted E4 coding region consists of the nucleotide sequence of SEQ ID NO: 23.

[0019] In one embodiment of the invention, the replicating recombinant adenovirus vector comprises a CMV promoter operably linked to a heterologous
15 nucleic acid sequence. In a particular embodiment, the CMV promoter has the nucleotide sequence of SEQ ID NO: 51.

[0020] According to embodiments of the invention, a replicative capacity of a replicating recombinant adenovirus vector of the invention is attenuated as compared to a replicative capacity of a wild-type human adenovirus serotype 26 or serotype 35.
20 In particular embodiments, the replicative capacity of a replicating recombinant adenovirus vector of the invention is attenuated by at least about 80-fold to 100-fold, as compared to the replicative capacity of a wild-type human adenovirus serotype 26 or 35.

[0021] In a particular embodiment, the invention provides a replicating
25 recombinant adenovirus vector comprising a recombinant human adenovirus serotype 26 genome having:

- (a) a promoter operably linked to a heterologous nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 48 or SEQ ID NO: 50;
- (b) a functional E1 coding region encoding the amino acid sequences of SEQ
30 ID NOs: 14, 15 and 16;
- (c) a partially deleted E3 coding region consisting of the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 6; and
- (d) a partially deleted E4 coding region consisting of the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 22.

[0022] In an embodiment of the invention, the replicating recombinant adenovirus vector comprises the heterologous nucleic acid sequence located between a left ITR and the 5'-end of the functional E1 coding region encoding the amino acid sequences of SEQ ID NOs: 14, 15, and 16.

5 [0023] In another particular embodiment of the invention, a replicating recombinant adenovirus vector comprises a recombinant human adenovirus serotype 26 genome having:

- (a) a CMV promoter having the nucleotide sequence of SEQ ID NO: 51 operably linked to a heterologous nucleic acid sequence comprising the
10 nucleotide sequence of SEQ ID NO: 47 or SEQ ID NO: 49;
- (b) a functional E1 coding region comprising the nucleotide sequence of SEQ ID NO: 13;
- (c) a partially deleted E3 coding region consisting of the nucleotide sequence of SEQ ID NO: 5; and
- 15 (d) a partially deleted E4 coding region consisting of the nucleotide sequence of SEQ ID NO: 23,
wherein the heterologous nucleic acid sequence is located between left ITR and 5'-end of the functional E1 coding region.

[0024] In another general aspect, the invention provides a composition comprising
20 a replicating recombinant adenovirus vector according to an embodiment of the invention and a pharmaceutically acceptable carrier, preferably the vector is isolated. In one embodiment, a composition of the invention is formulated for oral administration to a subject. In another embodiment, a composition of the invention is an enteric-coated capsule.

25 [0025] In yet another general aspect, the invention provides a method of producing a replicating adenovirus particle. The method comprises introducing a replicating recombinant adenovirus vector according to an embodiment of the invention into a cell under conditions sufficient for replication of the recombinant adenovirus genome of the vector and packaging of the adenovirus particle in the cell;
30 and collecting the adenovirus particle.

[0026] Other general aspects of the invention relate to a method of producing an immune response in a subject, and a method of vaccinating a subject against an infection comprising administering to the subject an immunogenically effective amount of a composition comprising a pharmaceutically acceptable carrier and a

replicating recombinant adenovirus vector according to the invention. Preferably, the composition is orally administered to the subject.

[0027] According to embodiments of the invention, a method of producing an immune response in a human subject or vaccinating a human subject against an HIV infection comprises orally administering to the subject an immunogenically effective amount of a composition comprising a pharmaceutically acceptable carrier and a replicating recombinant adenovirus vector comprising a recombinant serotype 26 adenovirus genome.

[0028] In one embodiment of a method of producing an immune response in a human subject or vaccinating a human subject against an HIV infection, the composition administered to the subject comprises a replicating recombinant adenovirus vector comprising a recombinant serotype 26 adenovirus genome having:

- (a) promoter operably linked to a heterologous nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 48 or SEQ ID NO: 50;
- (b) a functional E1 coding region encoding the amino acid sequences of SEQ ID NOs: 14, 15, and 16;
- (c) a partially deleted E3 coding region consisting of the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 6; and
- (d) a partially deleted E4 coding region consisting of the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 22.

[0029] Preferably, in the method of producing an immune response in a human subject or vaccinating a human subject against an HIV infection according to an embodiment of the present invention, the heterologous nucleic acid sequence is located between left ITR and 5'-end of the functional E1 coding region

[0030] In another embodiment of a method of producing an immune response in a human subject or vaccinating a human subject against an HIV infection, the composition administered to the subject comprises a replicating recombinant adenovirus vector comprising a recombinant serotype 26 adenovirus genome having:

- (a) a CMV promoter having the nucleotide sequence of SEQ ID NO: 51 operably linked to a heterologous nucleic acid sequence comprising the nucleotide sequence of SEQ ID NO: 47 or SEQ ID NO: 49;
- (b) a functional E1 coding region comprising the nucleotide sequence of SEQ ID NO: 13;

(c) a partially deleted E3 coding region consisting of the nucleotide sequence of SEQ ID NO: 5; and

(d) a partially deleted E4 coding region consisting of the nucleotide sequence of SEQ ID NO: 23

5 wherein the heterologous nucleic acid sequence is located between left ITR and 5'-end of the functional E1 coding region.

[0031] The invention also relates to a replicating recombinant adenovirus vector according to the invention for use in producing an immune response in a subject, or for use in the vaccination of a subject against an infection. Any of the replicating
10 recombinant adenovirus vectors according to the invention, including but not limited to those described herein, can be used in producing an immune response in a subject, or in the vaccination of a subject against an infection. Preferably, the replicating recombinant adenovirus vector according to the invention is for use in producing an immune response in a human subject or vaccinating a human subject against an HIV
15 infection.

BRIEF DESCRIPTION OF THE DRAWINGS

[0032] The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the
20 appended drawings. It should be understood that the invention is not limited to the precise embodiments shown in the drawings.

[0033] In the drawings:

[0034] Figure 1 shows schematic representations of a wild-type adenovirus genome, a genome of a replication-incompetent recombinant adenovirus vector, a
25 genome of replication-competent recombinant adenovirus vector containing an E1 coding region after the transgene cassette according to an embodiment of the invention, and a genome or a replication-competent recombinant adenovirus vector containing an E1 coding region before the transgene cassette according to another embodiment of the invention;

30 [0035] Figure 2 is a schematic representation of a cloning strategy for constructing an adaptor Ad26 plasmid vector that can be used to produce a replicating recombinant Ad26 vector according to embodiments of the invention; the plasmid vector (pAdApt26.E1atg.Empty; SEQ ID NO: 3) is designed to contain part of the

Ad26 genome, including the E1 coding region which is located downstream of the transgene cassette;

[0036] Figure 3 shows a schematic representation of an adaptor Ad26 plasmid vector and cosmid vector for producing a replicating recombinant Ad26 vector according to embodiments of the invention; (A): adaptor Ad26 plasmid vector (AdApt26.26E1.Mos1-HIVEnv; SEQ ID NO: 72) containing a heterologous nucleic acid sequence encoding the mosaic HIV antigen Mos1-HIVEnv in the transgene cassette cloned upstream of the E1 coding region (which encodes E1A, E1B 19K and E1B 55K proteins); (B): cosmid vector (pWeAd26.pIX-rITR.dE3.dE4.26ORF6; SEQ ID NO: 2) containing a partially deleted E3 coding region (E3-12.2K), a partially deleted E4 coding region where all E4 open reading frames have been deleted except for E4 open reading frame 6/7 (E4 Orf6/7), and the remaining portion of the Ad26 genome; the adaptor Ad26 plasmid vector shown in (A) and the cosmid vector (B) contain overlapping regions of nucleic acid sequence (marked as “Overlap with cosmid” and “Overlap with AdApter”, respectively) that facilitate homologous recombination in a host cell to produce a replicating recombinant Ad26 vector according to embodiments of the invention;

[0037] Figure 4 depicts gel images from the PCR and Western Blot analysis of plaques from the first round of purification of a replicating recombinant Ad26 vector according to an embodiment of the invention, rcAd26.Mos1-HIVEnv; at the top of the lanes, “+” indicates positive control, “-” and E indicate the negative controls, the rest of the lanes are labeled with the identifying number of the plaque tested, in 4(A) and 4(B) “M” represents the 1kb size marker (NEB, numbers indicate the size in kb, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0 and 10.0 kb), and in 4(C) “M” represents Magic marker (Invitrogen, numbers indicate the size in kD); (A): assessment of transgene region integrity by PCR using primers CMV.fwd (Ad26_1) (SEQ ID NO: 73) and E1.rev (Ad26_7) (SEQ ID NO: 74) (expected size of PCR product: 3.3 kb); (B) identity PCR for the E1 coding region using primers polyA.fwd (Ad26_8) (SEQ ID NO: 75) and Ad26.pIX.rev (Ad26_9) (SEQ ID NO: 76) (expected size of PCR product: 3 kb), the E3 coding region using primers Ad26.E3.fwd (Ad26_3) (SEQ ID NO: 77) and Ad26.E3.rev (Ad26_4) (SEQ ID NO: 78) (expected size of PCR product: 0.5kb), and the E4 coding region (primers Ad26.E4.fwd (Ad26_5) (SEQ ID NO: 79) & AdE4.rev (Ad26_6) (SEQ ID NO: 80)) (expected size of PCR product: 1.5kb); (C) analysis of Mos1-HIVEnv expression (140 kDa) by

Western blot using primary antibody anti-HIV-1 gp120 (cat# NEA-9301, Virus Research Products) and secondary antibody Goat anti-mouse IgG-HRP (cat# 170-6516, Biorad);

[0038] Figure 5 depicts images from PCR and Western Blot analysis of plaques from the second round of purification of a replicating recombinant Ad26 vector according to embodiments of the invention, rcAd26.Mos1-HIVEnv; on the top of the lanes “+” indicates positive control, “-” indicates negative control, and “U” indicates uninfected A549 cells, the rest of the lanes are labeled with the identifying number of the plaque tested; “M” indicates 1 kb marker (NEB, numbers indicate the size in kb) in 5(A) and 5(B), and Magic marker (Invitrogen, numbers indicate the amount of kD) in 5(C); (A): PCR analysis of the E1 coding region (primers polyA.fwd (Ad26_8) (SEQ ID NO: 75) and Ad26.pIX.rev (Ad26_9) (SEQ ID NO: 76)), E3 coding region (primers Ad26.E3.fwd (Ad26_3) (SEQ ID NO: 77) and Ad25.E3.rev (Ad26_4) (SEQ ID NO: 78) and E4 coding region (primers Ad26.E4.fwd (Ad26_5) (SEQ ID NO: 79) & Ad26.E4.rev (Ad26_6) (SEQ ID NO: 80); (B): PCR analysis of transgene region integrity using primers CMV.fwd (Ad26_1) (SEQ ID NO: 73) and Ad26_10 (SEQ ID NO: 81)); (C): Western Blot analysis of protein expression of mosaic HIV antigen (Mos1-HIVEnv) using primary antibody anti-HIV-1 gp120 (cat# NEA-9301, Virus Research Products) and secondary antibody Goat anti-mouse IgG-HRP (cat# 170-6516, Biorad);

[0039] Figure 6 compares the *in vitro* replication and infectivity reported as percent cytopathic effect (CPE) of replicating recombinant Ad26 vectors according to embodiments of the invention containing either (i) a deletion in the E3 coding region and lacking a transgene (rcAd26.dE3.empty), (ii) a deletion in the E3 and E4 coding regions and lacking a transgene (rcAd26.dE3.dE4.empty), (iii) a deletion in the E3 coding region and containing a transgene (rcAd26.dE3.Mos1Env), (iv) a deletion in the E3 coding region and E4 coding region, and containing a transgene (rcAd26.dE3.Mos1Env), and (v) wild-type Ad26 (Ad26.WT) in various cell lines; replication and infectivity was also compared to replication incompetent recombinant Ad26 vectors non-rcAd26.dE3.empty (deletion in E1 coding region, deletion in the E3 coding region, no transgene) and non-rcAd26.dE3.Mos1Env (deletion in E1 coding region, deletion in E3 coding region, containing a transgene); (A): *in vitro* infectivity in A549 cells (human, non-complementing); (B): *in vitro* infectivity in HuTu 80 cells

(human, non-complementing); (C): *in vitro* infectivity in PER.55K cells (human, complementing);

[0040] Figure 7 compares the *in vitro* replication and infectivity reported as percent cytopathic effect (CPE) of replicating recombinant Ad26 vectors according to
 5 embodiments of the invention in human cell lines and rhesus cell lines; (A): *in vitro* infectivity in A549 cells (human, non-complementing) and PER.55K (human, complementing) of Ad26.WT, rcAd26.dE3.empty, and replication-incompetent vector non-rcAd26.dE3.empty; (B) *in vitro* infectivity in rhesus monkey kidney cells (MK-2 cell line) of Ad26.WT, rcAd26.dE3.dE4.empty, rcAd26.dE3.dE4.Mos1Env, and
 10 replication-competent simian Ad vector derived from rhesus monkeys (rcSAd.SIVgag); the replication-competent simian Ad vector rcSAdSIVgag is labeled with an asterik (*);

[0041] Figure 8 shows the *in vitro* replication and infectivity reported as percent cytopathic effect (CPE) of replicating recombinant adenovirus vectors according to
 15 embodiments of the invention in human cell lines after two passages in cell culture; (A): *in vitro* infectivity of Ad26.WT, rcAd26.dE3.Empty, rcAd26.dE3.dE4.Empty, rcAd26.dE3.Mos1Env, and rcAd26.dE3.dE4.Mos1Env in A549 (human, non-complementing) cells after 1st reinfection and 2nd reinfection; (B) *in vitro* infectivity of Ad26.WT, rcAd26.dE3.Empty, rcAd26.dE3.dE4.Empty, rcAd26.dE3.Mos1Env, and rcAd26.dE3.dE4.Mos1Env in HuTu80 (human, noncomplementing) cells after 1st
 20 reinfection and 2nd reinfection; (C): comparison of viral titers for Ad26.WT, rcAd26.dE3.dE4.Empty, and rcAd26.dE3.dE4.Mos1Env lysates harvested at full CPE from cultures infected with each vector at an MOI of 1000; lysates from either infected A549 or HuTu80 cells were compared by performing a plaque assay in both
 25 A549 and PER.55K cells;

[0042] Figure 9 compares *in vitro* replication and infectivity reported as percent cytopathic effect (CPE) of replicating recombinant Ad26 vector rcAd26.dE3.dE4.Mos1Env and an Ad4-based vector expressing influenza H5 (rcAd4.H5), which has previously proven safe and immunogenic in phase 1 clinical
 30 trials; replication and infectivity was also compared to wild-type Ad26 (Ad26.WT) and Ad4 (Ad4.WT) vectors; the replicating recombinant Ad26 vector rcAd26.dE3.dE4.Mos1Env is pointed to by an arrow; (A): *in vitro* infectivity in A549 cells (human, non-complementing); (B) *in vitro* infectivity in HuTu80 cells (human, non-complementing); and (C) *in vitro* infectivity in PER.55K cells;

[0043] Figure 10 shows the results of IFN•-ELISPOT assay used to assess the immunogenicity of each of the replicating recombinant rcAd26 vectors according to embodiments of the invention (rcAd26.Mos1ENV; rcAd26.Mos2ENV; rcAd26Mos1GagPol; rcAd26Mos2GagPol) in Balb/C mice; results are reported as spot forming cells (SFC) per 10^6 splenocytes;

[0044] Figure 11 shows immunogenicity of lyophilized rcAd26.Mos1Env vector (injected with 10^8 or 10^9 virus particles) and binding antibody titers to HIV-1 Clade C envelope protein and Mosaic envelope protein in Balb/C mice; (A): mouse immunogenicity as determined by IFN•-ELISPOT assay using Mos1ENV and HIV PTE Env peptide pools; (B): binding antibody titers to HIV-1 Clade C envelope and Mosaic Env as determined by ELISA prior to dosing with rcAd26.Mos1Env vector and 28 days after immunization;

[0045] Figure 12 is a table of the results from RT-PCR performed on mouse serum, oral swab and rectal swab samples taken at days 0, 7, 14, 21, and 28 after intramuscular (IM) or intranasal (IN) immunization with either a replication incompetent recombinant Ad26 vector (Ad26.Mos1Env) (made replication incompetent by deletion in E1 coding region) or replication-competent recombinant Ad26 vector according to the invention (rcAd26.Mos1Env), with both vectors containing a nucleic acid sequence encoding the mosaic HIV antigen Mos1-HIVEnv; results are reported in copies/mL and control samples were spiked with 5.04×10^6 copies/mL of plasmid DNA; “***” indicates that no sample was taken;

[0046] Figure 13 shows the binding antibody titers in mice serum after IM or IN immunization with replication-incompetent recombinant Ad26 vector (Ad26.Mos1Env) and replication-competent recombinant Ad26 vector (rcAd26.Mos1Env) as determined by ELISA; (A): binding antibody titers to HIV-1 Clade C envelope protein; (B): binding antibody titers to Mosaic Env protein;

[0047] Figure 14 depicts the response to peptide pools in Balb/C Mice four weeks post-immunization with either replication-incompetent recombinant Ad26 vector (Ad26.Mos1ENV) or replication-competent recombinant Ad26 vector according to the invention (rcAd26.Mos1ENV) administered intramuscularly (IM) or intranasally (IN) as determined by IFN•-ELISPOT; for each regimen tested, the peptide pools from left to right are Mos1 Env, Mos2 Env, PTE Env 1, PTE Env 2, and PTE Env 3;

[0048] Figure 15 shows immunogenicity and replication of replication-competent Ad26-SIVGag (containing E1 coding region, “E1(+)”) and replication-incompetent

Ad26-SIVGag (lacking E1 coding region, “E1(-)”) vectors in non-human primates (Indian-origin rhesus monkeys *Macaca Mulatta*); (A): results of IFN•-ELISPOT to determine immune response reported as spot forming cells (SFC) per 10^6 peripheral blood mononuclear cells (PMBC); for each vector tested, the data for weeks 0, 2, 24, 26, and 32 is shown from left to right, respectively; (B): RT-PCR results to assess virus replication; monkeys 415-08, 421-08, and 451-08 received replication-incompetent Ad26.SIVgag vector, and monkeys 427-08, 429-08, and 432-08 received replication-competent Ad26.SIVgag vector; RT-PCR results are reported as copies/mL, and control samples are spiked with 5.04×10^5 copies/mL of plasmid DNA;

[0049] Figure 16 is a schematic representation of the cloning strategy used to construct a recombinant Ad35 adaptor plasmid vector that can be used to produce a replicating recombinant Ad35 vector according to embodiments of the invention, containing a transgene cassette before the E1 coding region and after the E1 coding region; (A): cloning of replicating recombinant Ad35 vector

pAdApt35BSU.E1atg.Empty, which is designed to contain part of the Ad35 genome, including the E1 coding region located downstream of the transgene cassette; (B): cloning of replicating recombinant Ad35 vector pAdApt35BSU.E1btg.Empty (SEQ ID NO: 26), which is designed to contain part of the Ad35 genome, including the E1 coding region located upstream of the transgene cassette;

[0050] Figure 17 is an agarose gel image assessing recombinant adenovirus vector stability by PCR analysis of the transgene region; the vectors screened by PCR include rcAd26.dE3.Mos1HIVEnv (“dE3,” containing deletion in E3 coding region), rcAd26.dE3.dE4.Mos1HIVEnv (“dE3.dE4” containing deletions in both E3 and E4 coding regions), and as positive control AdApt26.E1Mos1HIVenv (“+” Ad26 adaptor plasmid with coding sequence for Mos1HIVEnv cloned into the transgene cassette of pAdApt26 – see Figure 2); five passages of the virus post-production (labeled 1, 2, 3, 4, and 5) were screened by PCR using primers CMV.fwd (Ad26_1) (SEQ ID NO: 73) and E1.rev (Ad26_7) (SEQ ID NO: 74) with an expected PCR product size of 3.3 kb.

DETAILED DESCRIPTION OF THE INVENTION

[0051] Various publications, articles and patents are cited or described in the background and throughout the specification; each of these references is herein incorporated by reference in its entirety. Discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is for

the purpose of providing context for the invention. Such discussion is not an admission that any or all of these matters form part of the prior art with respect to any inventions disclosed or claimed.

[0052] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention pertains. Otherwise, certain terms used herein have the meanings as set forth in the specification. All patents, published patent applications and publications cited herein are incorporated by reference as if set forth fully herein. It must be noted that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural reference unless the context clearly dictates otherwise.

[0053] As used herein, “subject” means any animal, preferably a mammal, most preferably a human, to whom will be or has been administered a composition or replicating recombinant adenovirus vector according to embodiments of the invention. The term “mammal” as used herein, encompasses any mammal. Examples of mammals include, but are not limited to, cows, horses, sheep, pigs, cats, dogs, mice, rats, rabbits, guinea pigs, monkeys, humans, etc., more preferably a human.

[0054] As used herein, the term “adenovirus,” abbreviated “Ad,” refers to viruses of the adenoviridae family. Adenovirus is a medium-sized (90-100 nm), nonenveloped icosahedral virus containing double-stranded DNA. The term “adenoviridae” refers collectively to adenoviruses of the genera *Atadenovirus*, *Aviadenovirus*, *Ichadenovirus*, *Mastadenovirus*, and *Siadenovirus*. “Adenovirus” includes, but is not limited to human, bovine, ovine, equine, canine, porcine, murine and simian adenovirus species. Human adenoviruses, i.e., adenoviruses that can infect humans, can be classified into subgenera, or species, A-G.

[0055] As used herein, “human adenovirus” collectively refers to all human adenoviruses of subgenera A-G as well as the individual serotypes thereof.

[0056] The term “adenovirus serotype” means the individual members of a viral genus that are defined and identified by their expression of at least one serotype-specific epitope. Currently, there are over 60 known immunologically different types of adenovirus that can cause human infection including, but not limited to, human adenovirus serotypes 1, 2, 3, 4, 4a, 5, 6, 7, 7a, 7d, 8, 9, 10, 11A, 11P, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 34a, 35, 35p, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, and 51.

[0057] Preferably, an adenovirus used in accordance with the invention is derived from adenovirus serotype 26 (Ad26) having a wild-type genome of SEQ ID NO: 1, or adenovirus serotype 35 (Ad35) having a wild-type genome of SEQ ID NO: 27.

Adenovirus 26 is part of subgroup D and adenovirus 35 is part of subgroup B.

5 [0058] The various regions of the adenovirus genome have been mapped and are understood by those skilled in the art (see, e.g., Fields et al., *Virology* Volume 2, chapters 67 and 68, 3rd Edition, Lippincott-Raven Publishers). The genomic sequences of the various adenovirus serotypes, as well as the nucleotide sequence of the particular coding regions of the adenovirus genome, are known in the art and can
10 be accessed e.g., from GenBank and NCBI. In general, adenovirus genomes contain replication-essential genes, whose gene functions are required for replication and are encoded by, for example, the adenoviral early regions (e.g., E1, E2, and E4 regions) and late regions (e.g., the L1-L5 regions). Adenovirus genomes also contain genes involved in viral packaging (e.g., the Iva2 gene), and virus-associated RNAs (e.g.,
15 VA-RNA1 and/or VA-RNA-2).

[0059] The term “inverted terminal repeat sequence” or “ITR” refers to the common usage of the term with respect to adenoviruses and includes all ITR sequences and variations thereof that are functionally equivalent. The ITRs are the sets of sequences (motifs) which flank the linear adenovirus genome on the 5’-end
20 (“left ITR” or “lITR”) and 3’-end (“right ITR” or “rITR”), and are necessary for replication of the adenovirus genome. There is a high degree of sequence conservation within the ITR sequences between adenoviruses of different serotypes. A replicating recombinant adenovirus vector according to the invention can comprise any known adenovirus ITR sequence.

25 [0060] As used herein, the term “early gene 1 coding region” or “E1 coding region” refers to the full-length nucleic acid sequence in a human adenovirus genome that is first transcribed following infection, and that encodes the three human adenovirus E1 proteins known to be important for replication of the viral genome: E1A protein, E1B-19K protein, and E1B-55K protein.

30 [0061] As used herein, “early gene 3 coding region” or “E3 coding region” refers to the full-length nucleic acid sequence in a human adenovirus genome that encodes the seven human adenovirus E3 proteins: E3 13.2K, E3 CR1-alpha, E3 19K, E3 CR1-beta, E3 CR1-gamma, E3 RID-beta and E3 14.7K. Most of the E3 proteins have immunomodulatory functions. The human adenovirus E3 coding region is

dispensable for viral replication in tissue culture. However, some of the E3 proteins may be involved in the evasion of host immune defenses, and deletion of some or all of the E3 coding region may induce stronger pro-inflammatory responses in animal models (Sparer et al., 1996, *J. Virol.* 70: 2431–2439).

5 [0062] As used herein, “early gene 4 coding region” or “E4 coding region” refers to the full-length nucleic acid sequence in a human adenovirus genome that encodes at least the five human adenovirus E4 proteins: E4 orf1, E4 orf2, E4 orf3, E4 orf4 and E4 orf6/7. Adenovirus vectors lacking the E4 coding region may not be effective in delivery and long term retention of transgene expression under all circumstances
10 (Leppard, *Journal of General Virology* (1997), 78, 2131–2138.).

[0063] The terms “deleted” and “deletion” as used herein with respect to a coding region of a nucleic acid sequence, such as an E3 or E4 coding region of an adenovirus genome, mean that at least one nucleotide is omitted from the full-length wild-type nucleotide sequence. Deletions can be greater than about 1, 10, 50, 100, 200, or even
15 500 nucleotides. Deletions in the relevant coding region of the adenovirus genome can be about 1%, 5%, 10%, 25%, 50%, 75%, 80%, 90%, 99% or more of the coding region. Alternatively, the entire coding region can be deleted, meaning that all the nucleotides of the relevant coding region are omitted. A coding region that is “partially deleted” or “partly deleted” means that nucleotides of a portion of the
20 coding region that is less than the entire coding region are omitted.

[0064] As used herein, the term “heterologous” in the context of nucleic acid sequences, amino acid sequences, and antigens refers to nucleic acid sequences, amino acid sequences, and antigens that are foreign and are not naturally found associated with a particular adenovirus. A “heterologous nucleic acid sequence” can
25 be any transgene. As used herein, a “transgene” broadly refers to any gene or genetic material isolated from one source, either natural (e.g., cell) or synthetic (e.g., genetically engineered in a vector, recombinant DNA), and transferred to another source.

[0065] As used herein, the term “transgene cassette” refers to a region of a nucleic acid vector that contains a promoter and a multiple cloning site. The transgene
30 cassette is designed such that a heterologous nucleic acid sequence can be cloned into the multiple cloning site and placed under control of the promoter region. A transgene cassette does not necessarily have to contain a transgene or heterologous nucleic acid sequence, and can be “empty,” meaning that it lacks a transgene or

heterologous nucleic acid sequence. One of ordinary skill in the art will recognize that a transgene cassette can contain additional genetic regulatory elements, e.g., transcription termination signals, etc.

[0066] The term “recombinant adenovirus” refers to an adenovirus whose genome
5 has been modified through conventional recombinant DNA techniques. As used
herein, the term “recombinant adenovirus vector” refers to a vector construct
comprising nucleotide sequences derived from an adenovirus genome and optionally,
one or more heterologous nucleic acid sequences. According to embodiments of the
invention, a recombinant adenovirus vector comprises adenoviral nucleotide
10 sequences that are modified such that the recombinant adenovirus vector is replication
competent, but the replication efficiency is attenuated as compared to the replication
efficiency of the corresponding wild-type adenovirus. In accordance with this
embodiment, a recombinant adenovirus vector can be engineered to comprise a
mutated adenovirus genome by introducing one or more mutations in an adenovirus
15 genome, e.g., introducing deletions in one or more coding regions for adenoviral
proteins.

[0067] As used herein, “replicating recombinant adenovirus vector” and
“replication competent recombinant adenovirus vector” refer to a recombinant
adenovirus vector that can replicate or propagate upon introduction into a non-
20 complementing cell. In one particular embodiment of the invention, a “replicating
recombinant adenovirus vector” or “replication competent recombinant adenovirus
vector” can replicate or propagate upon introduction into a non-complementing
human cell. The terms “replicate” and “propagate” are used interchangeably, referring
to the ability of the adenovirus vector to reproduce or proliferate. These terms are well
25 understood in the art. The propagation or replication of a viral vector can be measured
using assays standard in the art and described herein, such as a burst assay or plaque
assay.

[0068] As used herein “replicative capacity” refers to the relative ability of a
recombinant adenovirus vector to replicate or propagate in a non-complementing cell.

30 [0069] As used herein, a “non-complementing cell” refers to a cell that does not
supply a particular genetic element, e.g., gene product, protein, etc. *in trans*. When
referring to a recombinant adenovirus vector, wherein the coding region for a
particular viral gene product is fully or partially deleted on the vector such that one or
more of the functional viral gene products cannot be expressed by the vector, a non-

complementing host cell specifically refers to a cell that does not supply the deleted functional gene product *in trans*. For example, if the E1 gene of the adenovirus genome is deleted to produce a recombinant adenovirus vector, a cell that does not supply a functional E1 gene *in trans* would be considered a non-complementing host cell. According to embodiments of the invention, a non-complementing cell is a human cell. Examples of non-complementing human cells include, but are not limited to, HuTu 80 cells (duodenum carcinoma, ATCC #HTB-40) and A549 cells (lung carcinoma, ATCC #CCL-185).

[0070] As used herein the terms “replication deficient,” “replication incompetent” and “non-replicating,” when used with reference to a recombinant adenovirus vector, are all intended to refer to a recombinant vector comprising a recombinant adenovirus genome that lacks certain genetic information necessary for replication and formation of a genome-containing capsid in a non-complementing cell. Replication deficient, replication incompetent and non-replicating recombinant vectors cannot replicate under physiological conditions either *in vivo* or *in vitro*, unless the missing viral genetic elements necessary for replication are provided by a second source, e.g., a complementing host cell.

[0071] As used herein, the term “operably linked” is to be taken in its broadest reasonable context, and refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid sequence is “operably linked” when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if it affects the transcription of the coding sequence.

[0072] The term “promoter” is used in its conventional sense, and refers to a nucleotide sequence that initiates the transcription of an operably linked nucleotide sequence. A promoter is located on the same strand near the nucleotide sequence they transcribe. Promoters may be naturally occurring or synthetic. When the vector to be employed is a viral vector, the promoters can be endogenous to the virus or derived from other sources.

[0073] The terms “attenuated” and “attenuation” refer to reduced pathogenicity and replicative capacity of a recombinant adenovirus vector as compared to the corresponding wild-type adenovirus. In one embodiment, attenuation refers to preventing replication in mammalian (e.g., human) cells. In another embodiment, attenuation refers to reducing, but not eliminating, replicative capacity of the virus,

such that it can replicate in mammalian cells, but to a lesser degree as compared to the corresponding wild-type virus. In yet another embodiment, attenuation refers to the inability to cause disease e.g., viral infection, or to cause disease, but to a lesser extent than wild-type virus. Attenuation can be achieved by using a variety of methods known in the art. For example, serial passage of viruses in animals, eggs, or tissue culture can lead to the acquisition of a variety of mutations that can result in reduced pathogenicity and replicative capacity. Attenuation can also be achieved by the complete or partial deletion of nucleic acid sequence from the genome of the virus, e.g., complete or partial deletions of E1, E3, or E4 coding regions of the adenovirus genome. Virus attenuation can be measured by any method known in the art, e.g., by the virus titer required to infect cells, RT-PCR, or the time required to achieve maximum cytopathic effect (CPE).

[0074] As used herein, the term “infection” refers to the invasion of a host by a disease causing agent. A disease causing agent is considered to be “infectious” when it is capable of invading a host, and replicating or propagating within the host. Examples of infectious agents include viruses, e.g., HIV and certain species of adenovirus, prions, bacteria, fungi, protozoa and the like.

[0075] In one general aspect, the invention provides a replicating recombinant adenovirus vector comprising a recombinant adenovirus genome. According to embodiments of the invention, a replicating recombinant adenovirus vector is replication competent, meaning that it can replicate in a non-complementing human cell. This is in contrast to many other known recombinant adenovirus vectors that have been used for vaccines, which are typically replication incompetent, thus cannot replicate or propagate in a non-complementing human cell.

[0076] The replicative capacity of a replicating recombinant adenovirus vector according to embodiments of the invention in a non-complementing cell is attenuated, or reduced, as compared to the replicative capacity of an otherwise identical wild-type adenovirus. According to embodiments of the invention, the attenuation in replicative capacity of a replicating recombinant adenovirus vector relative to the wild-type adenovirus can be between 2-fold and 1000-fold lower than the replicative capacity of the corresponding wild-type adenovirus. For example, the replicative capacity of the recombinant adenovirus vector can be decreased by 2-fold, 5-fold, 10-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, 100-fold, 150-fold, 200-fold, 250-fold, 500-fold, 750-fold or 1000-fold relative to the replicative capacity of the wild-type adenovirus

the replicating recombinant adenovirus vector is derived from. Preferably, the replicative capacity of the recombinant adenovirus vector is about 50-fold lower, and more preferably about 80-fold to about 100-fold lower than the replicative capacity of the wild-type adenovirus the replicating recombinant adenovirus vector derived from.

5 [0077] According to embodiments of the invention, a replicating recombinant adenovirus vector comprises a recombinant adenovirus genome. The recombinant adenovirus genome comprises (a) a promoter operably linked to a heterologous nucleic acid sequence; (b) a functional E1 coding region; (c) a deletion in the E3 coding region; and (d) a deletion in the E4 coding region, provided that E4 open
10 reading frame 6/7 is not deleted. See, e.g., Figure 1, which is a schematic representation of a replicating recombinant adenovirus vector according to an embodiment of the invention as compared to a wild-type adenovirus genome and non-replicating recombinant adenovirus vector lacking an E1 coding region. By deleting or partially deleting the E3 and E4 coding regions and retaining a functional E1
15 coding region in the recombinant adenovirus genome, the replicative capacity of the vector was attenuated as compared to wild-type, but the recombinant adenovirus vector was still replication competent. See, e.g., Figure 6.

[0078] According to embodiments of the invention, the recombinant adenovirus genome is derived from the genome of human adenovirus serotype 26 or the genome
20 of human adenovirus serotype 35, and preferably human adenovirus serotype 26.

[0079] In one embodiment of the present invention, a wild-type human adenovirus serotype 26 genome, such as that having the nucleotide sequence of SEQ ID NO: 1, comprises, *inter alia*, the E1 coding region (SEQ ID NO: 13), the E3 coding region (SEQ ID NO: 4), and the E4 coding region (SEQ ID NO: 17). The E1 coding region
25 encodes three proteins: E1A protein (SEQ ID NO: 14); the E1B 19K protein (SEQ ID NO: 15); and the E1B 55K protein (SEQ ID NO: 16). The E3 coding region encodes 7 proteins: E3 13.2K (SEQ ID NO: 6); E3 CR1-alpha (SEQ ID NO: 7); E3 19K (SEQ ID NO: 8); E3 CR1-beta (SEQ ID NO: 9); E3 CR1-gamma (SEQ ID NO: 10); E3
RID-beta (SEQ ID NO: 11); and E3 14.7K (SEQ ID NO: 12). The E4 coding regions
30 encodes 5 proteins: E4 ORF1 (SEQ ID NO: 18); E4 ORF2 (SEQ ID NO: 19); E4 ORF3 (SEQ ID NO: 20); E4 ORF 4 (SEQ ID NO: 21); and E4 ORF6/7 (SEQ ID NO: 22).

[0080] Likewise, in another embodiment of the present invention, a wild-type human adenovirus serotype 35 genome, such as that having the nucleotide sequence

of SEQ ID NO: 24, comprises, *inter alia*, the E1 coding region (SEQ ID NO: 36); the E3 coding region (SEQ ID NO: 27); and the E4 coding region (SEQ ID NO: 40). The E1 coding region encodes three proteins: E1A protein (SEQ ID NO: 37); the E1B 19K protein (SEQ ID NO: 38); and the E1B 55K protein (SEQ ID NO: 39). The E3 coding region encodes 7 proteins: E3 13.2K (SEQ ID NO: 29); E3 CR1-alpha (SEQ ID NO: 30); E3 19K (SEQ ID NO: 31); E3 CR1-beta (SEQ ID NO: 32); E3 CR1-gamma (SEQ ID NO: 33); E3 RID-beta (SEQ ID NO: 34); and E3 14.7K (SEQ ID NO: 35). The E4 coding regions encodes 5 proteins: E4 ORF1 (SEQ ID NO: 42); E4 ORF2 (SEQ ID NO: 43); E4 ORF3 (SEQ ID NO: 44); E4 ORF 4 (SEQ ID NO: 45); and E4 ORF6/7 (SEQ ID NO: 46).

[0081] Deletion of the E1 coding region from the Ad26 or Ad35 genome results in a replication incompetent recombinant adenovirus genome, meaning that the virus is unable to replicate in a non-complementing cell. Thus, a replicating recombinant adenovirus vector according to the invention comprises a functional E1 coding region.

[0082] The term “functional E1 coding region” is intended to encompass a nucleic acid sequence on the adenovirus vector that encodes active forms of E1A protein, E1B-19K protein, and E1B-55K protein, sufficient for viral replication. According to embodiments of the invention, a recombinant adenovirus vector can be engineered such that the functional E1 coding region is transferred to a different location within the recombinant viral vector as compared to the location of the E1 coding region in the wild-type adenovirus genome.

[0083] In one embodiment of the invention, a “functional E1 coding region” encodes active forms of E1A protein, E1B-19K protein, and E1B-55K protein, each of which has substantially identical activity as the wild-type human adenovirus E1A protein, E1B-19K protein, and E1B-55K protein, respectively.

[0084] In one embodiment of the invention, a functional E1 coding region of an Ad26 replicating recombinant adenovirus vector comprises a nucleotide sequence encoding Ad26 E1A protein (SEQ ID NO: 14), E1B-19K protein (SEQ ID NO: 15), and E1B-55K protein (SEQ ID NO: 16). In a particular embodiment of the invention, a functional E1 coding region of an Ad26 replicating recombinant adenovirus vector comprises the nucleotide sequence of SEQ ID NO: 13.

[0085] In another particular embodiment of the invention, a functional E1 coding region of an Ad35 replicating recombinant adenovirus vector comprises a nucleotide sequence encoding Ad35 E1A protein (SEQ ID NO: 37), E1B-19K protein (SEQ ID

NO: 38) and E1B-55K protein (SEQ ID NO: 39). In a particular embodiment of the invention, a functional E1 coding region of an Ad35 replicating recombinant adenovirus vector comprises the nucleotide sequence of SEQ ID NO: 36.

[0086] According to embodiments of the invention, a replicating recombinant adenovirus vector comprises a heterologous nucleic acid sequence that is located between a left ITR and the 5'-end of a functional E1 coding region. The heterologous nucleic acid sequence and the left ITR or the 5'-end of the E1 coding region can optionally be separated by a linker region of nucleic acid sequence. The present inventors surprisingly found that by placing the functional E1 coding region after the heterologous nucleic acid sequence, rather than before, both vector stability and expression of the heterologous nucleic acid sequence were increased, which was an unexpected effect.

[0087] According to embodiments of the invention, a replicating recombinant adenovirus vector comprises a deletion in the E3 coding region. A deletion in the E3 coding region can be, e.g., a partial deletion, such that the replicating recombinant adenovirus vector comprises a partially deleted E3 coding region.

[0088] According to a particular embodiment, in a partially deleted E3 coding region, all nucleic acid sequence of the E3 coding region with the exception of the nucleic acid sequence encoding the E3 12.5K protein product is deleted. When a recombinant adenovirus vector comprises a recombinant Ad26 genome, in one embodiment a partially deleted E3 coding region encodes the amino acid sequence of SEQ ID NO: 6, and in a particular embodiment, consists of the nucleotide sequence of SEQ ID NO: 5. When a recombinant adenovirus vector comprises a recombinant Ad35 genome, in one embodiment a partially deleted E3 coding region encodes the amino acid sequence of SEQ ID NO: 29, and in a particular embodiment, consists of the nucleotide sequence of SEQ ID NO: 28.

[0089] In certain embodiments of the invention, the E3 coding region of the recombinant adenovirus vector can be completely deleted. According to embodiments of the invention, a recombinant adenovirus vector with a completely deleted E3 coding region is replication competent, i.e., can replicate in a non-complementing human cell.

[0090] According to embodiments of the invention, a replicating recombinant adenovirus vector comprises a deletion in the E4 coding region, except E4 open reading frame 6/7. According to a particular embodiment, a replicating recombinant

adenovirus vector comprises a partially deleted E4 coding region, wherein all E4 open reading frames have been deleted except open reading frame 6/7. Thus, in a particular embodiment, when a recombinant adenovirus vector comprises a recombinant Ad26 genome, a partially deleted E4 coding region encodes the amino acid sequence of SEQ ID NO: 22, and consists of the nucleotide sequence of SEQ ID NO: 23. In another particular embodiment, when a recombinant adenovirus vector comprises a recombinant Ad35 genome, a partially deleted E4 coding region encodes the amino acid of SEQ ID NO: 41, and consists of the nucleotide sequence of SEQ ID NO: 46.

[0091] The present inventors have surprisingly found that a partial deletion of the E4 coding region, in addition to an at least partial deletion of the E3 coding region, produced a recombinant replicating adenovirus vector with increased stability, as compared to a recombinant replicating adenovirus vector containing the deletion of the E3 coding region and without the partial deletion of the E4 coding region. See, e.g., Figure 17. In the experiment for Figure 17, stability of the transgene of recombinant adenovirus vectors was determined by transgene PCR after 5 passages of the virus. A recombinant replicating adenovirus vector with both partial deletions of the E3 and E4 coding regions showed stability at all five passages of the virus post-production as determined by PCR analysis of the transgene region, whereas partial deletion of only the E3 coding region showed faint lower molecular weight bands at all passages post the first infection, indicating decreased stability of the transgene.

[0092] The present inventors have also surprisingly discovered that a replicating recombinant adenovirus vector according to the invention has attenuated replicative capacity. Attenuation of replicating adenovirus vectors comprising an E1 coding region containing both the E1B-19K protein and E1B-55K protein has not previously been demonstrated to the best of the knowledge of the inventors. Moreover, to the best of the knowledge of the inventors, attenuation of replicating Ad26 or Ad35 vectors has not previously been demonstrated. There is no a priori reason to predict that a construction of the recombinant adenovirus vector would have resulted in attenuated replicative capacity yet preserved stability. This achievement of attenuation and stability has practical value from a clinical, regulatory, and manufacturing perspective.

[0093] According to a preferred embodiment of the invention, a replicating recombinant adenovirus vector comprises a heterologous nucleic acid sequence located between the left ITR and the 5'-end of the E1 coding region, i.e., upstream of

the E1 coding region. A heterologous nucleic acid sequence for use in the invention can comprise any transgene, including but not limited to nucleic acid sequence useful for gene therapy, nucleic acid sequence encoding a therapeutically active protein or immunogenic polypeptide (e.g., antigen), and synthetic nucleic acid sequences
5 encoding genetically engineered or computationally designed protein or peptide sequences with potential therapeutic effects (e.g., mosaic antigens or proteins).

[0094] According to embodiments of the invention, a heterologous nucleic acid sequence encodes an immunogenic polypeptide or immunogen. In one embodiment, an immunogenic polypeptide or immunogen is any polypeptide or protein suitable for
10 protecting a subject (e.g., human) against a pathogenic disease or infection including, but not limited to, bacterial, protozoan, fungal, and viral diseases. In another embodiment, an immunogen or immunogenic peptide is any protein or polypeptide capable of inducing an immune response, e.g., a humoral and/or cellular mediated response, in a subject. In yet another embodiment, an immunogen or immunogenic
15 peptide is any protein or polypeptide that can be used to vaccinate a subject, i.e., produce immunity against a disease or infection in subject. In yet another embodiment, an immunogenic peptide is an antigen or portion thereof, or a combination of multiple antigens or portions thereof, that can induce an immune response or produce an immunity against a disease or infection in a subject.

20 [0095] Preferably, an immunogenic polypeptide is capable of raising in a host a protective immune response, e.g., inducing an immune response against a viral disease or infection, and/or produces an immunity in (i.e., vaccinates) a subject against a viral disease or infection, that protects the subject against the viral disease or infection. For example, the immunogen may be an orthomyxovirus immunogen (e.g.,
25 an influenza virus immunogen, such as the influenza virus hemagglutinin (HA) surface protein or the influenza virus nucleoprotein gene, or an equine influenza virus immunogen), or a lentivirus immunogen (e.g., an equine infectious anemia virus immunogen, a Simian Immunodeficiency Virus (SIV) immunogen, or a Human Immunodeficiency Virus (HIV) immunogen, such as the HIV or SIV envelope GP160
30 protein, the HIV or SIV matrix/capsid proteins, and the HIV or SIV gag, pol and env genes products). The immunogen can also be an arenavirus immunogen (e.g., Lassa fever virus immunogen, such as the Lassa fever virus nucleocapsid protein gene and the Lassa fever envelope glycoprotein gene), a poxvirus immunogen (e.g., vaccinia, such as the vaccinia L1 or L8 genes), a flavivirus immunogen (e.g., a yellow fever

virus immunogen or a Japanese encephalitis virus immunogen), a filovirus immunogen (e.g., an Ebola virus immunogen, or a Marburg virus immunogen, such as NP and GP genes), a bunyavirus immunogen (e.g., RVFV, CCHF, and SFS viruses), or a coronavirus immunogen (e.g., an infectious human coronavirus immunogen, such as the human coronavirus envelope glycoprotein gene, or a porcine transmissible gastroenteritis virus immunogen, or an avian infectious bronchitis virus immunogen). The immunogen can further be a polio immunogen, a herpes immunogen (e.g., CMV, EBV, HSV immunogens), a papillomavirus (e.g., HPV16 or HPV18) immunogen, a respiratory syncytial virus immunogen (such as F or G protein), a mumps immunogen, a measles immunogen, a rubella immunogen, a diphtheria toxin or other diphtheria immunogen, a pertussis immunogen, a hepatitis (e.g., hepatitis A or hepatitis B) immunogen, or any other vaccine immunogen known in the art in view of the present disclosure.

[0096] In a preferred embodiment, an immunogenic polypeptide induces an immune response or produces an immunity against human immunodeficiency virus (HIV). HIV is divided into two major types, HIV type 1 (HIV-1) and HIV type 2 (HIV-2). HIV-1 is the most common strain of HIV virus, and is known to be more pathogenic than HIV-2. HIV as used herein broadly refers to HIV type 1, HIV type 2, and subtypes thereof. In a preferred embodiment, an immunogenic polypeptide produces immunity against or induces an immune response against HIV-1.

[0097] According to embodiments of the invention, a heterologous nucleic acid sequence can encode an immunogenic polypeptide that is an HIV antigen. The HIV antigen can be an HIV-1 or HIV-2 antigen. Examples of HIV antigens include, but are not limited to *gag*, *pol*, and *env* gene products, which encode structural proteins and essential enzymes. *Gag*, *pol*, and *env* gene products are synthesized as polyproteins, which are further processed into multiple other protein products. The primary protein product of the *gag* gene is the viral structural protein gag polyprotein, which is further processed into MA, CA, SP1, NC, SP2, and P6 protein products. The *pol* gene encodes viral enzymes (Pol, polymerase), and the primary protein product is further processed into RT, RNase H, IN, and PR protein products. The *env* gene encodes structural proteins, specifically glycoproteins of the virion envelope. The primary protein product of the *env* gene is gp160, which is further processed into gp120 and gp41. Other examples of HIV antigens include gene regulatory proteins Tat and Rev; accessory proteins Nef, Vpr, Vif and Vpu; capsid proteins, nucleocapsid

proteins, and p24 viral protein. A heterologous nucleic acid sequence according to the invention can encode any HIV antigen, and preferably encodes a *gag*, *env*, and/or *pol* gene product, or portion thereof.

[0098] According to a preferred embodiment, a heterologous nucleic acid
5 sequence encodes an immunogenic polypeptide comprising an HIV Gag, Env, or Pol antigen, or any portion or combination thereof, more preferably an HIV-1 Gag, Env, or Pol antigen or any portion or combination thereof.

[0099] According to another preferred embodiment, a replicating recombinant
adenovirus vector according to the invention comprises a heterologous nucleic acid
10 sequence encoding a mosaic HIV antigen. As used herein, a “mosaic antigen” refers to a recombinant protein comprising fragments of natural sequences, and the recombinant protein is capable of eliciting immune responses against multiple naturally occurring antigenic or immunogenic determinants. The amino acid sequence of a “mosaic antigen” can be computationally generated and optimized using a genetic
15 algorithm. Mosaic antigens resemble natural antigens, but are optimized to increase the coverage of potential epitopes, more preferably T-cell epitopes, found in the natural sequences, thereby improving the breadth and coverage of the immune response. The term “mosaic antigen,” when used with respect to HIV, refers to a recombinant protein comprising fragments of natural sequences of HIV immunogens.

20 [0100] A mosaic HIV antigen according to embodiments of the invention is preferably a mosaic antigen comprising multiple immunogenic fragments from one or more of the HIV Gag, Pol, and/or Env polypeptide sequences, preferably from one or more of the Gag, Pol, and/or Env polypeptide sequences of HIV-1.

[0101] According to embodiments of the invention, a mosaic HIV antigen is
25 optimized to include a broader array of immunogenic sequences to increase coverage of epitopes, more preferably T-cell epitopes, found in circulating HIV strains.

[0102] In one embodiment, a mosaic HIV antigen according to the invention is a mosaic HIV antigen with multiple immunogenic sequences derived from one of Gag, Pol, and Env polypeptide sequences from one or more HIV types or subtypes,
30 preferably from one or more subtypes of HIV-1. For example, the mosaic HIV antigen can be a mosaic HIV Gag antigen with multiple immunogenic sequences derived from the sequences of HIV *gag* gene products; a mosaic HIV Pol antigen with multiple immunogenic sequences derived from the sequences of HIV *pol* gene

products; or a mosaic HIV Env antigen with multiple immunogenic sequences derived from the sequences of HIV *env* gene products.

[0103] In another embodiment, a mosaic HIV antigen according to the invention comprises a combination of immunogenic sequences derived from two of Gag, Pol, and Env polypeptide sequences from one or more HIV types or subtypes, preferably from one or more subtypes of HIV-1. Illustrative and non-limiting examples include a mosaic HIV Env-Pol antigen with epitopes derived from the sequences of HIV *env* and *pol* gene products; a mosaic HIV Env-Gag antigen with epitopes derived from the sequences of HIV *env* and *gag* gene products; a mosaic HIV Gag-Pol antigen with epitopes derived from the sequences of HIV *gag* and *pol* gene products; and a mosaic HIV Gag-Env antigen with epitopes derived from the sequences of HIV *gag* and *env* gene products.

[0104] In yet another embodiment, a mosaic HIV antigen according to the invention comprises a combination of immunogenic sequences derived from all three of Gag, Pol, and Env polypeptide sequences from one or more HIV types or subtypes, preferably from one or more subtypes of HIV-1.

[0105] Preferably, the mosaic HIV antigen is a mosaic HIV Env antigen, or a mosaic HIV Gag-Pol antigen.

[0106] In a particular embodiment of the invention, an immunogenic polypeptide is a mosaic HIV Env antigen comprising the amino acid sequence of SEQ ID NO: 48 (hereinafter referred to as “Mos1-HIVEnv”). In a more particular embodiment of the invention, a heterologous nucleic acid sequence of a replicating recombinant adenovirus according to the invention comprises the nucleotide sequence of SEQ ID NO: 47.

[0107] In another particular embodiment of the invention, an immunogenic polypeptide is a mosaic HIV Gag-Pol antigen comprising the amino acid sequence of SEQ ID NO: 50 (hereinafter referred to as “Mos1-HIVGagPol”). In a more particular embodiment of the invention, a heterologous nucleic acid sequence used in a replicating recombinant adenovirus according to the invention comprises the nucleotide sequence of SEQ ID NO: 49.

[0108] According to other particular embodiments of the invention, an immunogenic polypeptide is a mosaic HIV Env antigen comprising the amino acid sequence of SEQ ID NO: 69 (hereinafter referred to as “Mos2-HIVEnv”), or a mosaic HIV Gag-Pol antigen comprising the amino acid sequence of SEQ ID NO: 71

(hereinafter referred to as “Mos2-GagPol”). In more particular embodiments of the invention, heterologous nucleic acid sequences used in a replicating recombinant adenovirus according to the invention can comprise the nucleotide sequence of SEQ ID NO: 70 or SEQ ID NO: 72.

5 [0109] Studies of the T cell responses induced by previous vaccines using natural HIV sequences have shown that there is relatively limited breadth of recognition of epitopes on HIV, compared to the wide variety of circulating strains. For example, in the STEP trial, a median of approximately 1-2 epitopes per each of the Gag, Pol, and Nef proteins were recognized by each individual, but when this number is corrected
10 for epitope frequency in the pool of HIV to which a participant might have been exposed, the number would be considerably less [52]. Accordingly, the use of mosaic HIV antigens, and preferably mosaic HIV-1 antigens comprising multiple immunogenic fragments from one or more of HIV-1 Gag, Pol, and Env polypeptide sequences in a replicating recombinant adenovirus vector according to the invention
15 provides for increased breadth of recognition of epitopes, optimizing immunologic coverage of global HIV-1 virus diversity [53-55].

[0110] According to embodiments of the invention, a promoter is operably linked to a heterologous nucleic acid sequence. The promoter directs expression of the heterologous nucleic acid sequence within a eukaryotic cell, such as a mammalian or
20 human cell, and preferably in a non-complementing human cell. The promoter can be a mammalian promoter or a viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPRT), adenosine deaminase, pyruvate kinase, b-actin promoter and other constitutive
25 promoters. Inducible mammalian promoters include, but are not limited to, promoters that are strongly induced in the presence of environmental stimulus, such as a nutrient (e.g., sugar, amino acid), a change in temperature, pH, etc. Exemplary viral promoters which function in eukaryotic cells include, for example, promoters from the cytomegalovirus (CMV), simian virus (e.g., SV40), papilloma virus, adenovirus,
30 human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus, the long terminal repeats (LTR) of Moloney leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. The promoter can also be a tissue specific promoter that has activity in only certain cell types. Examples of tissue specific promoters include promoters specific to epithelial tissues, such as the

promoter of the E-cadherin gene or promoters of other genes that are specifically expressed in epithelial cells. Other promoters that can be used in the invention are known to those of ordinary skill in the art in view of the present disclosure.

[0111] Preferably, the promoter is a cytomegalovirus (CMV) promoter. In a particular embodiment, the promoter is a CMV promoter having the nucleic acid sequence of SEQ ID NO: 51.

[0112] In addition to the promoter, other regulatory sequences can also be included in the recombinant vector to regulate the expression of heterologous genes. Examples of such regulatory sequences include, but are not limited to, an enhancer, an upstream regulatory domain, a splicing signal, a polyadenylation signal, a transcriptional termination sequence, a translational regulatory sequence, a ribosome binding site and a translational termination sequence, etc.

[0113] Accordingly, the invention provides a novel adenovirus vector construct that is replication competent (see, e.g., Figures 6, 7, and 8), and can thus overcome certain disadvantages associated with replication incompetent vectors. A recombinant adenovirus vector according to the invention is replication competent, and can propagate in non-complementing human cells, albeit with lower efficiency than the wild-type adenovirus, thus amplifying transgene expression in the infected non-complementing host cells. Accordingly, transgene expression occurs not only from the recombinant adenovirus vectors present in the initial infecting virus, but also from additional copies of the recombinant adenovirus vectors that are produced by replication of the viral genome within the non-complementing host cell. This is in contrast to a replication-deficient recombinant adenovirus vector, such as an E1 deletion adenovirus vector, wherein all transgene expression is derived from the recombinant vectors present in the viral particles used for infection.

[0114] Replicating recombinant adenovirus vectors according to the invention can be produced by any method known in the art in view of the present disclosure. The nucleic acid sequences of the invention and other nucleic acids used to practice this invention, whether RNA, cDNA, genomic DNA, vectors, viruses or hybrids thereof, may be isolated from a variety of sources, genetically engineered, amplified, and/or expressed recombinantly. Any recombinant expression or propagation system can be used, including mammalian cells, bacterial, yeast, insect or plant systems.

[0115] Alternatively, these nucleic acids can be synthesized *in vitro* by well-known chemical synthesis techniques, as described in, e.g., Carruthers (1982) Cold

Spring Harbor Symp. Quant. Biol. 47:411-418; Adams (1983) J. Am. Chem. Soc. 105:661; Belousov (1997) Nucleic Acids Res. 25:3440-3444; Frenkel (1995) Free Radic. Biol. Med. 19:373-380; Blommers (1994) Biochemistry 33:7886-7896; Narang (1979) Meth. Enzymol. 68:90; Brown (1979) Meth. Enzymol. 68:109; Beaucage (1981) Tetra. Lett. 22:1859; U.S. Pat. No. 4,458,066. Double stranded DNA fragments can then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

[0116] Techniques for the manipulation of nucleic acids, such as, e.g., generating mutations in sequences, deleting portions of sequences, subcloning, labeling probes, sequencing, hybridization and the like are well described in the scientific and patent literature, see, e.g., Sambrook, ed., Molecular Cloning: A Laboratory Manual (2nd Ed.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989); Current Protocols in Molecular Biology, Ausubel, ed. John Wiley & Sons, Inc., New York (1997); Laboratory Techniques in Biochemistry and Molecular Biology: Hybridization with Nucleic Acid Probes, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993).

[0117] Nucleic acids, vectors, capsids, polypeptides, and the like can be analyzed and quantified by any of a number of general means well known to those of ordinary skill in the art. These include, e.g., analytical biochemical methods such as NMR, electron microscopy, spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography; various immunological methods, e.g. fluid or gel precipitation reactions, immunodiffusion, immuno-electrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), enzyme-linked immunosorbent spot (ELISPOT) assays, immuno-fluorescent assays; virus quantification assays, e.g., plaque forming unit (PFU) assays, focus forming assays; nucleic acid and protein analytical techniques, e.g., Southern analysis, Northern analysis, Western blot analysis, dot-blot analysis, gel electrophoresis (e.g., SDS-PAGE), RT-PCR, quantitative PCR, other nucleic acid or target or signal amplification methods, radiolabeling, scintillation counting, and affinity chromatography, etc.

[0118] Methods to generate and replicate viral vector constructs are well known in the scientific and patent literature, see, e.g., U.S. Pat. No. 5,981,225; U.S. Pat. No.

5,922,576; U.S. Pat. No. 5,880,102; Graham (1995) Mol. Biotechnol. 3:207-220, and for general methodologies, e.g., Sambrook, Ausubel, Tijssen. Viral genome genetic engineering, transformation and infection techniques in cell culture, viral manipulation and isolation techniques, Ad replication competent cell lines and permissive conditions for Ad replication, and the like, are all well known and described in the scientific and patent literature, see e.g., Krougliak (1995) Hum. Gene Ther. 6:1575-1586; Gorziglia (1999) J. Virol. 73:6048-6055; Cote (1998) Biotechnol. Bioeng. 59:567-575; Hartigan-O'Connor (1999) J. Virol. 73:7835-7841; U.S. Pat. No. 5,851,806; U.S. Pat. No. 5,880,102; U.S. Pat. No. 5,882,877; U.S. Pat. No. 5,891,690; U.S. Pat. No. 5,965,541; U.S. Pat. No. 5,981,225; U.S. Pat. No. 5,985,846; U.S. Pat. No. 5,994,106; U.S. Pat. No. 5,955,281.

[0119] For example, recombinant adenoviral vectors can be generated by a variety of known procedures, e.g., *in vivo* homologous recombination method (see, e.g., He (1999) Proc. Natl. Acad. Sci. USA 95:2509-2514; Aoki (1999) Mol. Med. 5:224-231; Souza (1999) Biotechniques 26:502-508; U.S. Pat. No. 5,919,676); by the *in vitro* direct ligation method (see, e.g., Mizuguchi (1998) Hum. Gene Ther. 9:2577-2583); or using circular adenoviral DNA (see, e.g., Tashiro (1999) Hum. Gene Ther. 10:1845-1852). As another technique, the altered sequences can be inserted in a bacterial clone taking advantage of a bacterial recombination system, e.g., as the method described by Chartier (1996) J. Virol 70:4805-4810. This system uses a bacterial plasmid that contains a full length copy of an Ad genome coupled with a simple gene replacement method in *E. coli*. This allows manipulation of any portion of the Ad genome in a prokaryotic or eukaryotic expression vector followed by insertion into a full length copy of an Ad genome. The full length Ad chromosome is cut once with a restriction enzyme in the region to be replaced. Bacteria are co-transformed with this linearized molecule. Homologous recombination yields a circular molecule that is competent for replication in the bacterial cell. Presence of the altered Ad sequence can be confirmed by PCR and Southern blotting.

[0120] Preferably, a recombinant adenoviral vector of the invention is obtained through homologous recombination in a host cell, the homologous recombination being between two linearized plasmids containing regions of sequence homology. In this method, a plasmid/cosmid vector system can be used (Figure 2). The plasmid contains part of the adenovirus genome, starting from the left end of the genome at the left ITR to a site within the pIX coding region. The plasmid includes a transgene

cassette containing a promoter, and a multiple cloning site (MCS) for insertion of a heterologous nucleic acid sequence under control of the promoter in the transgene cassette. One of ordinary skill in the art will appreciate that the transgene cassette can comprise other regulatory elements to control expression, stability, etc. of the heterologous nucleic acid sequence from the transgene cassette, e.g., signal sequences such as a polyadenylation (polyA) transcription termination signal. The adaptor plasmid also contains the E1 coding region, which according to embodiments of the invention, is downstream of the transgene cassette. The 3'-end of the adaptor plasmid contains nucleic acid sequence that is homologous to a portion of the cosmid vector sequence for facilitating homologous recombination, and is preferably about 2.0 to 2.5 kb. The cosmid vector contains the remaining majority of the adenovirus genome spanning from a site within the pIX coding region to the right ITR, including the E3 and E4 coding region. The region of homology with the adaptor plasmid is at the 5' end of the cosmid vector and includes the pIX sequence.

[0121] The adenovirus sequence can be manipulated, e.g., by deleting the E3 coding region, completely or partially, and partially deleting the E4 coding region, and the heterologous nucleic acid sequence cloned into the MCS of the adapter plasmid prior to homologous recombination of the plasmid and cosmid to obtain the recombinant adenovirus vector using any method known in the art in view of the present disclosure.

[0122] Any plasmid/cosmid system can be used to generate a recombinant adenovirus vector in view of the present disclosure. Cosmids are commercially available hybrid plasmids that contain the Lamda phage *Cos* sequence. Cosmids allow for stable insertion of large DNA fragments (up to approximately 50 kb), whereas other plasmids carrying a DNA fragment of this size can become unstable. The adenovirus 26 or adenovirus 35 genome can be cloned into the plasmid/cosmid using methods known in the art in view of the present disclosure. The adenovirus plasmid/cosmid, such as pAdApt26 or pAdApt35 plasmid, allows for insertion of heterologous nucleic acid sequences into the respective backbone of the adenovirus 26 or adenovirus 35 genome. The adenovirus plasmid/cosmid can also serve as the template or source for amplification or manipulation of Ad genes of interest. In accordance with the invention, recombinant adenovirus vector can be produced by homologous recombination upon co-transfecting an appropriate cell type with the plasmid vector, containing part of the adenovirus genome and the transgene cassette

with the inserted heterologous nucleic acid sequence, and the cosmid vector containing the remaining adenovirus genome. Co-transfection can be performed by any method known in the art, e.g., the DEAE dextran method (McCutchan and Pagano, 1968), the calcium phosphate procedure (Graham and van der Eb, 1973),
5 microinjection, lipofection (liposome transfection), electroporation, etc.

[0123] Amounts of plasmid and cosmid used in the co-transfection can vary depending on the particular plasmid and cosmid constructs, cell types, etc., and typically range between approximately 0.2 to 10 • g of DNA per 10⁶ cells. For example, a plasmid:cosmid ratio of 1:3 (e.g., 2 µg plasmid: 6 µg cosmid) can be used,
10 although the ratio of plasmid: cosmid can be adjusted as necessary to optimize the co-transfection. The plasmid and cosmid are linearized before transfection, e.g., by restriction enzyme digest. Cells suitable for transfection include any cell line permissive for adenovirus infection, including but not limited to HEK-293 cells, HeLa cells, 293-D22 cells, A549 cells, HuTu 80 cells, HCT-15 cells, IGROV-1 cells, U87
15 cells, W162 cells, PER.55K cells, and PER.C6 cells (Fallaux, et al., New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum Gene Ther* 9, 1909-1917,1998) or derivatives thereof. In certain embodiments, PER.C6 cells are used for transfection of plasmid/cosmid and subsequent generation of the recombinant adenovirus vector.

20 [0124] The transfected cells can be cultured in any suitable vessel known in the art. For example, cells can be grown and the infected cells can be cultured in a biogenerator or a bioreactor. Generally, “biogenerator” or “bioreactor” means a culture tank, generally made of stainless steel or glass, with a volume of 0.5 liter or greater, comprising an agitation system, a device for injecting a stream of CO₂ gas,
25 and an oxygenation device. Typically, it is equipped with probes measuring the internal parameters of the biogenerator, such as the pH, the dissolved oxygen, the temperature, the tank pressure or certain physicochemical parameters of the culture (for instance the consumption of glucose or of glutamine or the production of lactate and ammonium ions). The pH, oxygen, and temperature probes are connected to a
30 bioprocessor which permanently regulates these parameters. In other embodiments, the vessel is a spinner flask, a roller bottle, a shaker flask, or in a flask with a stir bar providing mechanical agitation.

[0125] The cells can be passaged, and incubated until the development of cytopathic effect (CPE), or the virus-containing cells can be frozen for storage and

subsequently used for re-infection of cells, or for virus isolation. The recombinant adenovirus vector can be isolated and purified by any method known in the art in view of the present disclosure, e.g, purification from plaques. Isolated and purified recombinant adenovirus vector can be stored in liquid form, e.g., aqueous buffer, and
5 frozen, or it can be lyophilized and stored in dry form.

[0126] The invention also provides a method of producing a replicating adenovirus particle. According to embodiments of the invention, the method comprises introducing a replicating recombinant adenovirus vector according to the invention into a cell under conditions sufficient for replication of the recombinant
10 adenovirus genome and packaging of the adenovirus particle in the cell. Thereafter, the adenovirus particles can be collected from the cell.

[0127] As used herein, “viral particle” or “virion” refers to a viral genome enclosed in a protein coat or shell. A viral particle is essentially an inert carrier of a viral genome. Viral particles are assembled inside cells from virus-specific
15 components and carry the viral genome from cell to cell e.g., by infection. Viral particles themselves are not capable of growth or replication, and thus serve as carriers of the viral genome. An “adenovirus particle” specifically refers to a viral particle containing a wild-type adenovirus genome, recombinant adenovirus genome, or recombinant adenovirus vector.

[0128] As used herein, the term “collecting,” with reference to the production of adenovirus particles, means the isolation of a population of recombinant virus particles from cells used to produce the viral particles. Viral particles can be collected from the virus-containing cells, from the growth medium of cells, or both. To collect viral particles from the cells used to produce the particles, the cells are lysed to release
25 the particles. Thereafter, the particles can be purified according to any of the methods known in the art and described herein.

[0129] Any appropriate cell line for propagating adenovirus vectors can be used in a method for producing an adenovirus particle in view of the present disclosure, including, but not limited to PER.C6 cells or HEK293 cells. The produced adenoviral particles can be collected from the cell culture supernatant or from the cells after lysis
30 (e.g., by chemical means, freeze/thawing, osmotic shock, mechanic shock, sonication and the like). Host cell DNA can be degraded by treatment with a DNase/RNase, such as Benzonase (American International Chemicals, Inc.). The viral particles can be isolated by consecutive rounds of plaque purification. The collected viral particles

can be purified using any suitable technique known in the art in view of the present disclosure (e.g., chromatographic methods, ultracentrifugation on a cesium chloride or sucrose gradient).

5 [0130] Alternatively, virus-containing cells, wherein a recombinant adenovirus vector was introduced into the cell, can be frozen prior to collecting the adenoviral particles. These cells can be used to propagate a new batch of cells for producing replicating adenoviral particles, or they can be stored and the adenoviral particles can subsequently be collected from the cells.

10 [0131] In another general aspect, the invention provides a composition comprising a replicating recombinant adenovirus vector according to the invention and a pharmaceutically acceptable carrier. Preferably, the replicating recombinant adenovirus vector is isolated. It will be appreciated that a pharmaceutical composition or vaccine comprising the recombinant adenovirus may contain adjuvants, excipients, and carriers.

15 [0132] According to embodiments of the invention, a composition can be a vaccine. As used herein, the term “vaccine” refers to a composition comprising a replicating recombinant adenovirus vector of the invention that can provide active acquired immunity to a particular disease. In a preferred embodiment, a vaccine is a composition comprising a replicating recombinant adenovirus vector of the invention
20 that can provide active acquired immunity to an HIV infection.

[0133] Compositions of the invention can be formulated in any matter suitable for administration to a subject to facilitate administration and improve efficacy, including, but not limited to, oral (enteral) administration and parenteral injections. The parenteral injections include intravenous injection or infusion, intra-arterial
25 injection, subcutaneous injection, intramuscular injection, and intra-articular injection. Compositions of the invention can also be formulated for other routes of administration including transmucosal, ocular, rectal, long acting implantation, sublingual administration, under the tongue, from oral mucosa bypassing the portal circulation, inhalation, or intranasal.

30 [0134] In a preferred embodiment, a composition is formulated for oral administration. Compositions suitable for oral administration include, but are not limited to, powders, capsules, caplets, gelcaps, granules, and tablets. In one preferred embodiment, enteric coated capsules or tablets are formulated for oral administration. Further detail may be found, e.g. in Remington's Pharmaceutical Sciences,” 1990,

18th ed., Mack Publishing Co., Easton, Pa. In another preferred embodiment, a composition is formulated for injection, e.g., intramuscular or subcutaneous, as a liquid preparation. Compositions suitable for injection include solutions, suspensions, and emulsions.

5 [0135] Oral vaccine compositions can be taken in the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders, containing from about 10% to about 95% of the active ingredient, preferably about 25% to about 70%. Oral and/or intranasal vaccination may be preferable to raise mucosal immunity (which plays an important role in protection against pathogens
10 infecting the respiratory and gastrointestinal tracts) in combination with systemic immunity. Excipients that can be included in oral formulations include, for example, pharmaceutical grades of mannitol, lactose, starch, sucrose, histidine, glycine, gelatin, magnesium stearate, sodium saccharin cellulose, magnesium carbonate, sodium chloride, magnesium chloride, and the like. Additional ingredients, such as alcohol,
15 detergent (e.g., Tween), ethylenediamine-tetraacetic acid (EDTA), can also be included in the oral composition.

[0136] Pharmaceutically acceptable carriers can include one or more excipients such as binders, disintegrants, swelling agents, suspending agents, emulsifying agents, wetting agents, lubricants, flavorants, sweeteners, preservatives, dyes, solubilizers and
20 coatings. Carriers can take a wide variety of forms depending on the form of preparation desired for administration. For liquid injectable preparations, for example, suspensions and solutions, suitable carriers and additives include water, glycols, oils, alcohols, preservatives, coloring agents and the like. For solid oral preparations, for example, powders, capsules, caplets, gelcaps and tablets, suitable
25 carriers and additives include starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like. For nasal sprays/inhalant mixtures, the aqueous solution/suspension can comprise water, glycols, oils, emollients, stabilizers, wetting agents, preservatives, aromatics, flavors, and the like as suitable carriers and additives.

30 [0137] A vaccine composition can be prepared from freshly harvested viral cultures by methods that are standard in the art. For example, the growth of the virus is monitored by standard techniques (observation of cytopathic effect, immunofluorescence or other antibody-based assays), and harvested when a sufficiently high viral titer has been achieved. The viral stocks can be further

concentrated or lyophilized by conventional methods before inclusion in the vaccine formulation. Other methods, such as those in described in Thomas, et al., Agri-Practice, V.7 No. 5, pp. 26-30., can also be employed.

[0138] According to embodiments of the invention, a recombinant adenovirus vector can be lyophilized prior to formulation for administration as either a solid preparation or liquid preparation. The present inventors have found that the design of a replicating recombinant adenovirus vector according to the invention increases the stability of the vector and allows the vector to survive lyophilization without decay of virus particles/plaque forming unit (vp/PFU) titers or reduced immunogenicity (Figure 11). The lyophilized vector can be packaged into capsules to obtain a solid preparation for oral administration. The lyophilized vector can also be resuspended in a suitable buffer to obtain a liquid preparation for parenteral administration.

[0139] According to embodiments of the invention, compositions for administration will commonly comprise a buffered solution in a pharmaceutically acceptable carrier, e.g., an aqueous carrier such as buffered saline and the like. The compositions can also contain pharmaceutically acceptable substances as required to approximate physiological conditions such as pH adjusting and buffering agents.

[0140] Accordingly, the stability of a replicating recombinant adenovirus vector according to the invention in lyophilized form allows the vector to be packaged into capsules, tablets, and other solid preparations suitable for oral administration. Thus, compositions of the invention are easy to administer and allow for simple, efficient delivery to a subject.

[0141] According to an embodiment of the invention, the composition is a capsule comprising a purified recombinant vector, such as rcAd26.Mos1Env, rcAd26.Mos2Env, rcAd26.Mos1GagPol and/or rcAd26.Mos2GagPol; a buffer, such as Tris buffer, phosphate buffer; one or more salts, such as NaCl, MgCl₂; a nonionic surfactant, such as a polysorbate, e.g., Tween 20, Tween 40, Tween 60 or Tween 80; a bulking agent, such as lactose, sucrose, or hydroxypropyl methylcellulose (HPMC); and one or more additional ingredients such as EDTA, histidine, lactate, mannitol, ethanol, etc. The composition is buffered at a pH of 6.0 and 8.0, preferably 6.5 to 7.5, more preferably 7.0 to 7.4. In a particular embodiment, the composition is enteric-coated. The composition can be stored frozen, for example at a temperature of less than -65°C.

[0142] In another general aspect, the invention provides a method of producing an immune response in a subject. According to embodiments of the invention, the method comprises administering to a subject an immunogenically effective amount of a composition comprising a replicating recombinant adenovirus vector of the invention and a pharmaceutically acceptable carrier. Any of the replicating recombinant adenovirus vectors described herein and compositions thereof can be used in a method of producing an immune response in a subject according to the invention.

[0143] The invention also relates to a method of vaccinating a subject, e.g., human. A method of vaccination can be against an infection, e.g., bacterial, protozoan, fungal and viral diseases, etc., and is preferably against a viral infection, more preferably an HIV infection. A method of vaccination according to the invention comprises administering to the subject an immunogenically effective amount of a composition comprising a pharmaceutically acceptable carrier and a replicating recombinant adenovirus vector. Any of the replicating recombinant adenovirus vectors described herein and compositions thereof can be used in a method of vaccination according to the invention.

[0144] As used herein, "HIV infection" specifically refers to the invasion of a host organism, such as the cells and tissues of the host organism, by the HIV virus.

[0145] As used herein, "vaccinating" or "vaccination" means to produce an immune response or immunity against a disease or infection (bacterial, viral, etc.) in a subject, e.g., human. According to embodiments of the invention, a composition of the invention is administered to vaccinate a subject against an HIV infection.

[0146] As used herein, "an immunogenically effective amount" means an amount of a composition sufficient to induce a desired immune effect or immune response in a subject. In one embodiment, an immunogenically effective amount means an amount sufficient to induce an immune response in a subject. In another embodiment, an immunogenically effective amount means an amount sufficient to produce immunity in a subject, e.g., provide a protective effect against a disease such as a viral infection. An immunogenically effective amount can vary depending upon a variety of factors, such as the physical condition of the subject, age, weight, health, etc.; route of administration, such as oral or parenteral; the particular application, whether inducing immune response or providing protective immunity; the specific replicating recombinant adenovirus vector administered; the immunogen encoded by the

replicating recombinant adenovirus vector, and the particular disease, e.g., viral infection, for which immunity is desired. An immunogenically effective amount can readily be determined by one of ordinary skill in the art in view of the present disclosure.

5 [0147] As general guidance, an immunogenically effective amount can range from about 10^8 viral particles to about 10^{12} viral particles, for example 10^8 , 10^9 , 10^{10} , 10^{11} , or 10^{12} viral particles. An immunologically effective amount can be administered in a single composition, or in multiple compositions, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 compositions (e.g., tablets or capsules), wherein the administration
10 of the multiple capsules collectively provides a subject with the immunologically effective amount. It is also possible to administer a immunogenically effective amount to a subject, and subsequently administer another dose of an immunogenically effective amount to the same subject, in a so-called prime-boost regimen. This prime-boost regime is well known to s a person of ordinary skill in the vaccine field. Further
15 booster administrations can optionally be added to the regimen, as needed.

[0148] According to preferred embodiments, in a method of producing an immune response or a method of vaccination, a composition according to the invention is administered orally, preferably as a solid composition (tablet, capsule, etc.), to a human subject. However in some embodiments, a composition is administered by
20 injection in liquid formulation, e.g., intramuscular injection.

[0149] According to embodiments of the invention, a replicating recombinant adenovirus vector elicits an immune response against an HIV infection. An immune response can be a cellular response or a humoral response. In general, cellular response refers to the activity of the CD4 and CD8+ T cells, whereas humoral
25 response refers to antibody production and activity. More particularly, a cellular immune response includes a response that enables host CD8+ T cells to limit replication of HIV and kill HIV-infected cells, thus dampening or eliminating a first local, muscosal focus of HIV infection during the early stages of infection.

[0150] The ability to induce or stimulate an anti-HIV immune response upon
30 administration in an animal or human organism can be evaluated either *in vitro* or *in vivo* using a variety of assays which are standard in the art. For a general description of techniques available to evaluate the onset and activation of an immune response, see for example Coligan et al. (1992 and 1994, Current Protocols in Immunology; ed J Wiley & Sons Inc, National Institute of Health). Measurement of cellular immunity

can be performed by measurement of cytokine profiles secreted by activated effector cells including those derived from CD4+ and CD8+ T-cells (e.g. quantification of IL-10 or IFN gamma-producing cells by ELISPOT), by determination of the activation status of immune effector cells (e.g. T cell proliferation assays by a classical [³H] thymidine uptake), by assaying for antigen-specific T lymphocytes in a sensitized subject (e.g. peptide-specific lysis in a cytotoxicity assay).

[0151] The ability to stimulate a humoral response can be determined by antibody binding and/or competition in binding (see for example Harlow, 1989, Antibodies, Cold Spring Harbor Press). For example, titers of antibodies produced in response to administration of a composition providing an immunogen can be measured by ELISA. ELISPOT can also be used to assess humoral immune response to identify and enumerate the number of cells secreting an antibody produced in response to administration of an antigen.

[0152] According to embodiments of the invention, upon administration to a subject, a replicating recombinant adenovirus vector expresses an immunogenic polypeptide. Any of the immunogenic polypeptides described herein can be encoded by a recombinant viral vector and administered to a subject in a method of the invention. The expressed immunogenic polypeptide is presented to the immune system of the subject, thereby inducing the required response to produce immunity, or induce an immune response to treat or prevent a disease or infection. For example, the response can be the production of antibodies specific to the immunogenic polypeptide.

[0153] Preferably, upon administration to a subject, a replicating recombinant adenovirus vector expresses a mosaic HIV antigen. Presentation of a mosaic HIV antigen according to the invention to the immune system of a subject can induce the production of antibodies specific to the HIV, such as antibodies specific to HIV *gag*, *pol*, and/or *env* gene products, depending on the sequence composition of the expressed mosaic HIV antigen.

[0154] According to preferred embodiments of the invention, in a method of producing an immune response in a subject or vaccination of a subject, a replicating recombinant adenovirus vector encodes a mosaic HIV antigen comprising the amino acid sequence of SEQ ID NO: 48 (Mos1-HIVEnv) or SEQ ID NO: 50 (Mos1-HIVGagPol). A replicating recombinant adenovirus vector according to the invention

can also encode a mosaic HIV antigen comprising the amino acid sequence of SEQ ID NO: 69 (Mos2-HIVEnv) or SEQ ID NO: 71 (Mos2-GagPol).

[0155] According to embodiments of the invention, compositions comprising replicating recombinant adenovirus vectors can be administered to a subject prior to
5 an HIV infection, or after the onset of an HIV infection. When administered prior to an HIV infection, e.g., for vaccination of a subject, an immune response can be induced that provides protective immunity to the subject, effectively immunizing the subject to any future HIV infection. When administered after the onset of an HIV infection, an immune response can be induced that treats the HIV infection, e.g., by
10 destroying HIV infected cells, preventing the HIV virus from replicating, and/or inhibiting viral entry of the HIV virus into cells.

[0156] Compositions of the invention can be administered in a variety of dosage forms, and dosing regimens. The appropriate dosing regimen, including the frequency of administration, mode of administration, and the immunogenically
15 effective amount, will vary, and can be affected by the particular condition of the patient (e.g., age, health, condition), time of administration (e.g., prior to infection or after the onset of infection), etc. One of ordinary skill in the art would readily be able to determine the appropriate dosing regimen in view of the present disclosure. For example, in a method of vaccinating a subject, compositions can be administered in
20 two doses, an initial dose and a second dose, wherein the second dose is administered several days, weeks or months after the initial dose. As another non-limiting and illustrative example, in a method of producing an immune response, a daily or weekly dosing regimen can be established, wherein a determined immunogenically effective amount of the composition is administered once daily, or once weekly, for a set period
25 of time, e.g., several weeks or months. The dosing regimen can be adjusted accordingly depending on the response of the subject, e.g., improvement or worsening in condition.

[0157] Without wishing to be bound by any theories, it is hypothesized that a replicating recombinant adenovirus vector of the invention can induce immune
30 responses that differ in their location and cellular phenotype from replication-incompetent, parenteral (injectable) vaccines. This is thought to be in part due to the stability of the recombinant adenovirus vector to lyophilization, thus allowing the recombinant vector to be administered orally as a solid composition. Specifically, replicating recombinant adenovirus vectors of the invention are believed to induce

potent immune responses in the Gut-Associated Lymphoid Tissue (GALT), which is an early target for HIV infection and destruction of CD4+ cells [56]. Thus, the replicating recombinant adenovirus vectors may function by preventing or limiting HIV replication on first entry.

5 [0158] The following examples of the invention are to further illustrate the nature of the invention. It should be understood that the following examples do not limit the invention and that the scope of the invention is to be determined by the appended claims.

10

EXAMPLES

EXAMPLE 1: Cloning of empty Ad26 adaptor plasmid

(pAdApt26.E1atg.Empty) with E1 coding region after transgene cassette.

[0159] The empty Ad26 recombinant vector, pAdAp26.E1atg.Empty (SEQ ID
15 NO: 3) was constructed as shown in Figure 2. The pAdApt26.E1atg.Empty vector contains the E1 coding region after the transgene cassette. “Empty” denotes that the vector does not contain a heterologous nucleic acid sequence or transgene, however any heterologous nucleic acid sequence of interest can be inserted into the multiple cloning site of the transgene cassette of the pAdApt26.E1atg.Empty vector under
20 control of the CMV promoter, and upstream of the E1 coding region.

Production of “PCR Fragment A” and “PCR Fragment B”

[0160] Two polymerase chain reactions (PCRs) were set up. The first PCR was to amplify the E1 coding region from the wild-type adenovirus vector genome (“Ad26
25 WT”), and was performed using the following primer pair: forward primer, Ad26WT.463.MluI.fwd
5’-CACAGACGCGTATCAGCTGATCCGCAGGGTATTTA-3’ (SEQ ID NO: 54);
and reverse primer, Ad26WT.XhoI.rev 5’-CTGGGCATGTAGCTCGAGGCCAGT-3’
(SEQ ID NO: 55). The forward primer was designed to introduce an MluI site at the
30 start of the E1 coding region, and the reverse primer was designed to overlap with the existing XhoI in the pIX region of the Ad26 viral genome. This resulted in a PCR product flanked by a MluI and a XhoI site (“PCR fragment A”).

[0161] The second PCR was to amplify part of the transgene cassette from the pAdApt26 vector (Abbink et al. Comparative seroprevalence and immunogenicity of

six rare serotype recombinant adenovirus vaccine vectors from subgroups B and D. *J. Virology* (2007) 81(9), 4654-4663) and was performed using the following primer pair: forward primer, AdApt26.NdeI.fwd primer 5'-

GTGTATCATATGCCAAGTACGCCC-3' (SEQ ID NO: 52); and reverse primer,

5 AdApt26.MluI.rev

5'-CGATCACGCGTATCTAGACATGATAAGATACATTGATG-3' (SEQ ID NO:

53). The forward primer was designed to overlap with the existing NdeI site in the

CMV promoter of the pAdApt26 vector, and the reverse primer was designed to

introduce a MluI site immediately after the polyA sequence. This resulted in a PCR

10 product flanked by a NdeI and a MluI site ("PCR fragment B").

Cloning of AdApt26.E1atg.Empty (E1 region put after transgene cassette)

[0162] PCR fragment A was digested with NdeI and MluI, and PCR fragment B

was digested with MluI and XhoI. pAdApt26 vector was digested with NdeI and

15 XhoI. All fragments were gel purified. A triple ligation was set up with these digested

fragments and clones were screened by restriction enzyme analysis and sequencing to

confirm pAdApt26.26E1.empty was obtained.

EXAMPLE 2: Cloning of Ad26 adaptor plasmid containing a heterologous

20 **nucleic acid sequence encoding a mosaic HIV antigen (pAdApt26.26E1.Mos1-HIVEnv).**

[0163] The Ad26 adaptor plasmid containing a heterologous nucleic acid

sequence encoding the mosaic HIV antigen Mos1HIVEnv (SEQ ID NO: 48) cloned

into the transgene cassette under control of the CMV promoter,

25 pAdApt26.26E1.Mos1-HIVEnv (Figure 3A) was constructed as follows.

[0164] Mos1-HIVEnv was digested from a plasmid that was synthetically

generated by GeneART® that contained the Mos1-HIVEnv transgene (SEQ ID NO:

47) in a bacterial backbone. KpnI and BamHI sites were designed flanking the

transgene sequence. pAdApt26.26E1.Empty was digested with KpnI and BamHI.

30 After purification, both DNA fragments were ligated together using T4 DNA ligase.

Clones were sequenced to verify integrity.

EXAMPLE 3: Cloning of Ad26 cosmid vector (pWe.Ad26.pIX-rITR.dE3dE426orf6).

[0165] The Ad26 cosmid vector pWe.Ad26.pIX-rITR.dE3dE426orf6 (SEQ ID NO: 2) (Figure 3B) containing a partially deleted Ad26 E3 coding region (SEQ ID NO: 5) and a partially deleted Ad26 E4 coding region (SEQ ID NO: 22) was constructed as follows.

Cloning of pWe/Ad26.pIX-rITR.dE3.26orf6 Ad26 Cosmid (Ad26 cosmid containing native Ad26E4orf6)

10 [0166] The E4orf6 region of adenovirus serotype 5 (Ad5E4orf6) present in the cosmid vector pWe/Ad26.pIX-rITR.dE3.5orf6 (Abbink et al. 2007, *supra*) was first replaced with the native E4orf6 region of adenovirus 26 (Ad26E4orf6). A PCR fragment was generated from the *EcoRI* site present in the Ad26 fiber protein coding region to the *MluI* site in the E4orf3 of Ad26. Ad26 WT was used as the template for the PCR reaction, with the following primer pair was used: forward primer, Ad26.E4orf6.fwd 5'-CTATTTGATGAGAATGGAATTCTATTA-3' (SEQ ID NO: 56); and reverse primer, Ad26.E4orf6.rev 5'-CTTATGCTGGATGTACGCGTAGAG-3' (SEQ ID NO: 57). This PCR fragment was ligated into the pWe/Ad26.pIX-rITR.dE3.5orf6 cosmid (digested with *EcoRI* and partially with *MluI*) thereby replacing the Ad5orf6 part to provide pWe/Ad26.pIX-rITR.dE3.26orf6.

25 Cloning of pWe/Ad26.pIX-rITR.dE3.dE4.26orf6 (Ad26 cosmid vector containing partially deleted E3 coding region and a partially deleted E4 coding region)

[0167] Two PCR fragments were generated. The first was generated starting at the *EcoRI* site in the Ad26 fiber protein coding region until the start of the E4orf6 region with a designed *MluI* site at the start of the E4orf6 using the following primer pair: forward primer, Ad26.dE4.EcoRI-MluI.fwd 5'-GACTGCTACTACAAAGAAGGATGTA-3' (SEQ ID NO: 58); and reverse primer Ad26.dE4.EcoRI-MluI.rev 5'-TATTCAACGCGTAGTACGACAAGGTACGCAAGAGAAT-3' (SEQ ID NO: 59). pBr/Ad26.dE3.26orf6 (Abbink et al. 2007, *supra*) was used as the template.

[0168] The second PCR fragment was generated at the start of the E4orf1 with a designed MluI site until the SrfI site using the following primer pair: forward primer, Ad26.dE4.MluI-SrfI.fwd5'-

TATTCAACGCGTAGCTCAGCCCGCTTACCAGTAGA-3' (SEQ ID NO: 60); and

5 reverse primer, Ad26.dE4.MluI-SrfI.rev 5'-GCGTCTGGCGCGGCGCAGCAGA-3' (SEQ ID NO: 61). pBr/Ad26.dE3.26orf6 was used as the template.

[0169] Both of the generated PCR fragments were digested with EcoRI/MluI or MluI/SrfI. pBr/Ad26.dE3.26orf6 was digested with EcoRI/SrfI. Before the E4orf6 was swapped with that of Ad5, this plasmid had its native Ad26 E4orf6 gene still

10 present. The EcoRI/MluI and MluI/SrfI digested PCR fragments were ligated into the digested pBr/Ad26.dE3.26orf6 by triple ligation, providing pBr/Ad26.dE3.dE4.26orf6, in which E4orf1 through E4orf4 were deleted.

[0170] Finally, the pBr/Ad26.dE3.dE4.26orf6 and the pBr/Ad26.SfiI (Abbink et al., 2007, supra) plasmids were digested with SrfI and PacI and ligated into a pWe
15 cosmid backbone that was digested with PacI resulting in pWe/Ad26.pIX-rITR.dE3.dE4.26orf6 cosmid vector.

EXAMPLE 4: Production of a Replicating Recombinant Adenovirus 26 Vector rcAd26.Mos1-HIVEnv.

20 [0171] The adaptor plasmid pAdApt26.26E1.Mos1-HIVEnv obtained in Example 2, and the cosmid vector pWe.Ad26.pIX-rITR.dE3dE426orf6 obtained in Example 3 (Figure 3) were first cleaned and amplified to ensure that the plasmid and cosmids were free of any animal components. The plasmids were cleaned with chaotropic salt, which is present in the NT buffer in the Nucleospin Extract II kit of Macherey-Nagel.
25 These cleaned plasmids were electroporated into MegaX DH10B bacteria, which were subsequently grown on animal component free LB agar containing 50 µg/mL ampicillin. After the electroporation, one single colony was isolated and a streak was performed on animal component free LB agar containing 50 µg/mL ampicillin. This streak was repeated one more time. After the second streak, three colonies were
30 grown in animal component free LB broth with 50 µg/mL ampicillin and subsequently tested by miniprep analysis. One culture was selected and used to inoculate a maxiprep culture, again using animal component free LB broth containing 50 µg/mL ampicillin. The plasmids were subsequently isolated using an Endotoxin free maxiprep kit of Macherey-Nagel. The RNase present in the kit was not used as it

is of animal origin. Instead the RNase T1 of Roche, which is derived from the fungus called *Aspergillus oryzae*, was used. The integrity of pAdApt26.26E1.Mos1-HIVEnv plasmid and pWe/Ad26.dE3.dE4.26orf6 cosmid vector were confirmed by restriction enzyme analysis.

5 [0172] The rcAd26.Mos1-HIVEnv replicating recombinant vector was generated by transient transfection. The DNA solution for transfection was produced by mixing 1 µg of linearized plasmid pAdApt26.26E1.Mos1-HIVEnv and 5 µg of linearized cosmid pWe/Ad26.dE3.dE4.26orf6.

[0173] To rescue the virus, Lipofectamine 2000CD was added to the DNA
10 mixture at a DNA:Lipofectamine 2000CD ratio of 1 µg : 2.5 µl, and incubated at room temperature for 30-40 min. PER.C6 cells were seeded in a T25 flask one day prior to transfection, washed with DMEM, and the transfection mixture was incubated on the cells for 4 hours at 37°C and 10% CO₂. After 4 hours, the transfection mixture was removed and culture medium was added. After incubation at 37°C and 10% CO₂
15 for 2 days, the transfected cells were passaged to a T75 flask and incubated until the development of cytopathic effect (CPE).

[0174] As CPE development did not progress to full CPE, the total crude material was harvested, stored at -20°C and subsequently used for reinfection of PER.C6 seeded in a T75 flask. By the reinfection process full CPE was obtained and the total
20 crude material was harvested and stored at -20°C. This material was used to perform the first plaque purification. A total of 12 plaques were picked from the highest dilutions and propagated on PER.C6 cells seeded in a 24 well plate. The propagated plaques were tested for integrity of the transgene region by PCR using the proofreading polymerase Pfu (Promega; cat# M7745) and primer set Ad26-1 (SEQ ID
25 NO: 73) and Ad26-7 (SEQ ID NO: 74) (see Figure 4). The PCR product includes the CMV promoter, polyA sequence, the start of the coding region of E1A protein, which has an expected size of 3.3 kb. Sequences were analyzed by alignment of the obtained transgene region sequences from PCR of the rcAd26.Mos1-HIVEnv vector to the reference sequence for the PCR product (SEQ ID NO: 93).

30 [0175] Plaque 3 was prioritized and subjected to a second round of plaque purification. Twenty plaques from the highest dilutions were selected from round two. A total of 12 plaques were propagated on PER.C6 cells seeded in a MW24 plate format and analyzed. Transgene region integrity for subsequent rounds of plaque purification was analyzed by PCR using the proofreading polymerase Pfu (Promega;

cat# M7745) and the transgene region primers Ad26-1 (SEQ ID NO: 73) and Ad26-10 (SEQ ID NO: 81) (located in the CMV promoter and E1 coding region, respectively), rather than Ad26-1 and Ad26-7, as it gave more specific product bands (see Figure 5). Alignment of the obtained transgene region sequences of the rcAd26.Mos1-HIVEnv vector was compared with the Mos1-HIVEnv reference sequence (SEQ ID NO: 93).

[0176] Based on the results obtained, plaque number 3 (see Figure 5B, lane 2) was selected to continue with. The virus was subsequently expanded, and the rcAd26.Mos1- HIVEnv vector was purified from the cell pellet by a three-step freeze/thaw cycle and a two-step cesium chloride (CsCl) centrifugation procedure.

The host cell DNA was degraded by Benzonase treatment. The vector was aliquoted into cryovials, snap frozen on liquid nitrogen and stored at -80°C.

EXAMPLE 5: Characterization of the rcAd26.Mos1 HIVEnv recombinant adenovirus vector.

Determination of infectious titer of the virus.

[0177] The viral particle (vp) amount (vp/mL) of the purified rcAd26.Mos1-HIVEnv was initially determined using spectrophotometric methods by measuring the optical density at 260 nm in the presence of 1% SDS. Alternatively, HPLC was also used to determine vp/mL. The infectious particle amount was determined by TCID50 assay using 911 cells, or plaque forming unit (PFU) assay. For the PFU assay, cells permissive to adenovirus growth, such as 293 or PER.55K cells, were seeded at day 1 in 6 well plates at a density of 8.5×10^5 cells per well. On day 0, the cells were infected with log dilutions of purified adenovirus vector followed by agar overlay after an infection period of 24 hours. At day 14, the plaques were counted in the wells in which the plaques were sufficiently separated so there was no overlap of multiple plaques. Virus particles per plaque forming unit (vp/PFU) was calculated as follows:

$$\# \text{ of plaques at day 14} / \text{dilution} = \text{PFU/ml}$$

Average of all dilutions counted on a plate is calculated.

$$(\text{Average PFU/ml}) / \text{viral titer (vp/ml)} = \text{vp/PFU}$$

Transgene PCR and Sequence Analysis

[0178] Transgene and PCR sequence analysis was performed as described above in Example 4.

5 Transgene Expression

[0179] Expression levels of the transgene encoding the Mos1-HIVEnv mosaic HIV antigen were determined by Western blot analysis. A549 cells were infected at increasing multiplicity of infections (MOIs: 1000, 2500, 5000, 10000, 25000 and 50000 vp per cell) with the obtained purified rcAd26.Mos1HIVEnv vector. After 3
10 days of incubation, lysates were prepared from the infected A549 cells and one uninfected control sample. Mos1-HIVEnv expression for rcAd26.Mos1HIVEnv was confirmed by Western blot analysis using a primary antibody anti-HIV-1 gp120 (Virus Research Products; cat# NEA-9301) and secondary antibody goat anti-mouse IgG-HRP (Biorad; cat# 170-6516).

15

Purity Determination

[0180] The purity of rcAd26.Mos1-HIVEnv was determined by SDS-PAGE analysis under denaturing conditions, which results in disintegration of the adenoviral particle and separation of the individual proteins that constitute the
20 rcAd26.Mos1HIVEnv vector. Four different vp amounts (5×10^8 vp, 1×10^9 vp, 3×10^9 vp and 6×10^9 vp) were analyzed with varying concentrations of bovine serum albumin (BSA) (0.5 μ g, 0.1 μ g, 0.05 μ g and 0.01 μ g).

[0181] The size of the observed protein bands was compared to the Novex Sharp Pre-stained Marker migration pattern. The apparent sizes were used to
25 putatively assign the bands on the gel to specific proteins. After instant blue staining (Expedeon; cat# 194-ISB1L), the gel was scanned and the intensity of the bands were determined by the Gel Pro 6.0 software. BSA was used as an internal marker of known concentration. The protein band pattern observed for the rcAd26.Mos1-HIVEnv was as expected for adenovirus, as the visible bands are comparable to
30 known adenoviral related proteins (118 kD: Hexon; 60 kD: Penton base; 59 kD: pIIIa (minor capsid protein); 45 kD: pV (minor core protein); 26 kD: pVI (minor capsid protein); 20 kD: pVII (core protein). No unexpected or unknown bands were detected.

EXAMPLE 6: *In Vitro* Infectivity and Growth of Ad26 vectors in various cell lines.

[0182] The infectivity and growth kinetics of replication competent Ad26 vectors (rcAd26) were analyzed. Specifically, the deletion of E3/E4 and insertion of the Mos1-HIVEnv transgene was assessed in terms of attenuation, as defined by the virus titer required to infect cells and the time to achieve maximum cytopathic effect (CPE) of replication-competent Ad26 vectors.

[0183] The rcAd26 vectors tested are shown below in Table 1, and the infectivity and growth kinetics of each of these vectors was compared to wild-type Ad26 (Ad26.WT) in the following cell lines: A549 (human epithelial lung carcinoma cell line; ATCC #CCL-185, Manassas, VA), HuTu 80 (human duodenum adenocarcinoma cell line; ATCC #HTB-40), and PER.55K (Human epithelial cell line that complements the Ad E1 region [12]). The rcAd26 vectors were made replication competent by adding the E1 region back into the replication-incompetent Ad26 behind the transgene cassette. Versions of the vector that were compared had either part of the E3 region deleted, or part of both the E3 and E4 region deleted, and were with or without the Mos1-HIVEnv transgene. For comparison, the replication incompetent version of wild-type Ad26 was also tested.

Table 1: Ad26 Vectors Used for *In Vitro* Infectivity and Growth Study

Vector	Replication Competent	E3 Deleted	E4 Deleted	Transgene
rcAd26.dE3.dE4.Mos1.Env	Yes	Yes	E4 orf 1-4	HIVMos1.Env
rcAd26.dE3.Mos1.Env	Yes	Yes	No	HIVMos1.Env
rcAd26.dE3.dE4.empty	Yes	Yes	E4 orf 1-4	No
rcAd26.dE3.empty	Yes	Yes	No	No
Ad26.WT	Yes	No	No	No
non-rcAd26.dE3.Empty	No	Yes	No	No
non-rcAd26.dE3.Mos1.Env	No	Yes	No	HIVMos1.Env

[0184] The infectivity assays were performed by first seeding A549, HuTu 80, or PER.55K cells into 6-well plates the day before infection. The next day, the cells were infected with the Ad26 vectors at various multiplicity of infection (MOI) (1000, 333, 100, 33, 10, and 0). The cultures were then monitored and scored daily for the percentage cytopathic effect (CPE) observed out to 6 days post-infection. Each vector

and cell line combination was tested in 3 replicate experiments. The results of the *in vitro* infectivity experiments for each of the tested cell lines are shown in Figure 6.

[0185] For all rcAd26 vectors, the *in vitro* replicative capacity was significantly reduced (required more virus to infect the monolayer and took longer to cause maximum CPE) in the non-complementing A549 cells (Figure 6A) and HuTu 80 cells (Figure 6B) as compared to wild-type Ad26 in both of these cell lines. Decreased *in vitro* replicative capacity occurred in a step-wise fashion with vectors containing only the partial E3 deletion having only a slight reduction, then becoming more reduced as both E3 and E4 were partially deleted. Replicative capacity was most reduced in vectors containing the transgene encoding Mos1-HIVEnv, with rcAd26.dE3.dE4.Mos1Env (partial E3/E4 deletion, and encoding Mos1-HIVEnv) having the most pronounced decrease in replicative capacity. Approximately 100-fold more virus was needed to achieve the same virus growth compared to wild-type Ad26. As expected, the non-replicating Ad26 vectors did not replicate in either the A549 or HuTu 80 cell lines.

[0186] As a control, the vector growth in E1-complementing PER.55K cells was evaluated. The data demonstrated that there was no significant difference in growth between the various Ad26 vectors as compared to wild-type when PER.55K cells were infected (Figure 6C).

[0187] The results of these experiments indicate that the replicating recombinant rcAd26 vectors had a significant decrease in infectious titer, and increase in time to achieve full CPE as compared to wild-type Ad26 when used to infect A549 and HuTu80 cell lines, but had no decrease in growth when used to infect PER.55K cells. The decreased *in vitro* replicative capacity of the replicating recombinant rcAd26 vectors in non-complementing cells was most pronounced with partial deletions of both the E3 and E4 coding regions, and when the vector contained a transgene, e.g., nucleic acid sequence encoding Mos1-HIVEnv. The replicative capacity of the rcAd26.Mos1Env vector was at least 100-fold less than the replicative capacity of wild-type Ad26. This reduction in replicative capacity (i.e., attenuation) had not been previously observed in other adenovirus vectors, and could not be predicted based on the structure of the vector construct.

EXAMPLE 7: Comparison of *in vitro* infectivity and growth of replicating recombinant Ad26 vectors in human cell lines and rhesus monkey cell lines.

[0188] The infectivity and growth kinetics of the replicating recombinant Ad26 vector rcAd26.dE3.Empty, and replication incompetent Ad26 vector non-

5 rcAd26.dE3.Empty, both as described above in Table 1, were analyzed in A549 cells and PER.55K cells. The infectivity assays were performed as described above in Example 6 by infecting cells at various MOIs (1000, 100, or 10). Wild-type Ad26 (Ad26.WT) was tested as a control. The results are reported as percent CPE, and are shown in Figure 7A.

10 [0189] The infectivity and growth kinetics of replicating recombinant Ad26 vectors rcAd26.dE3.dE4.Empty and rcAd26.dE3.dE4.Mos1Env were analyzed in MK-2 cells (rhesus monkey kidney cell line). For the controls, Ad26.WT and replication competent simian Ad vector derived from rhesus monkeys (rcSAd.SIVgag) were tested. The infectivity assays were performed also as described
15 above in Example 6 by infecting cells at MOIs of 1000, 333, and 100. The results are reported as percent CPE, and are shown in Figure 7B.

[0190] The results of the above experiments demonstrate that wild-type Ad26 and the replicating recombinant Ad26 vectors tested grew efficiently in A549 cells, whereas the replication-incompetent Ad26 vector did not, as expected (Figure 7A).
20 Moreover, all three vectors grew well in the adenovirus E1 protein complementing cell line, PER.55K cells, as expected. Additionally, the results confirm that replication of the replicating recombinant Ad26 vectors is species specific, as none of these vectors replicated in the rhesus monkey cell line MK2, whereas the replication competent simian Ad vector replicated well in MK-2 cells (Figure 7B).

25

EXAMPLE 8: *In vitro* infectivity and growth of replicating recombinant rcAd26 vectors after multiple cell passages.

[0191] To verify that the CPE observed above in Examples 6 and 7 indicated that the recombinant Ad26 vectors were indeed replicating viruses, the replication kinetics
30 of the replicating recombinant rcAd26 vectors rcAd26.dE3.Empty, rcAd26.dE3.dE4.Empty, rcAd26.dE3.Mos1Env, and rcAd26.dE3.dE4.Mos1Env (Table 1), were compared through two additional passages of the virus in either A549 or HuTu80 cell cultures.

[0192] Primary cell cultures were harvested at full CPE by harvesting both the cells and supernatant by pipetting and freezing at -20°C. Subsequent re-infections were performed by thawing, and then clarifying each lysate by centrifugation. To re-infect cells, 100 µL of each cell lysate was added to cells that had been seeded at 8.5×10^5 cell per well into a 6-well plate the day before. Cultures were then monitored and scored daily for percent CPE for 6 days post-infection, and infected cell lysates harvested the day of full CPE. PFU assays were performed by infecting either PER.55K cells or A549 cells that had been seeded into 6-well plates at a concentration of 8.5×10^5 cells per well the day before with serial dilutions of adenovirus. The next day, an agar overlay was added, and plaques were counted at day 7 and day 14 post infection. All serial dilutions were done in duplicate.

[0193] The results are shown in Figures 8A-8B, and demonstrate that all replication competent recombinant rcAd26 vectors tested replicated well through 2 passages in both A549 and HuTu80 cell lines, confirming that the CPE observed reflected vector replication, rather than some other effect, e.g., nonspecific cell lysis.

[0194] To determine the viral titer of the lysates harvested at full CPE in the *in vitro* infectivity assays, a plaque forming unit (PFU) assay was performed using the same samples as above. Lysates were harvested at full CPE from A549 and HuTu80 cell cultures infected with 1000 MOI of each vector, and then assessed for viral titers using A549 cells (non-complementing E1 cells). Experiments with PER.55K cells (E1 complementing cells) were performed as a control.

[0195] From the results of the PFU assays shown in Figure 8C, it can be seen that titers closely mimicked the results obtained in the *in vitro* infectivity assays, with attenuation occurring in a step-wise fashion. More specifically, the rcAd26.dE3.dE4.Empty vector had a PFU titer 1.9 logs lower than wild-type Ad26, and with the addition of the Mos1Env transgene (rcAd26.dE3.dE4.Mos1Env), the titer decreased to 2.7 logs lower than the wild-type virus. This effect was even more pronounced with lysates from HuTu80 cells, in which the rcAd26.dE3.dE4.Empty vector had a titer 2.2 logs lower than wild-type Ad26, and with addition of the transgene, the titer dropped to 4.4 logs lower than the wild-type virus. In the control experiments with PER.55K cells, no significant differences were observed among the vectors tested, as expected.

EXAMPLE 9: Comparison of *in vitro* infectivity of replicating recombinant Ad26 vectors and replicating recombinant Ad4 vector.

[0196] Experiments were performed using the *in vitro* infectivity assay to
5 compare the infectivity of the rcAd26.dE3.dE4.Mos1Env vector to that of the
replicating recombinant adenovirus 4 (Ad4)-based vector expressing influenza H5,
which is the hemagglutinin protein from H1N1 (rcAd4.H5) in A549, HuTu80, and
PER.55K cells. The rcAd4.H5 vector, developed by PaxVax, has previously proven
safe and immunogenic in a Phase 1 clinical trial [1, 59]. The rcAd4.H5 and wild-type
10 Ad4 (Ad4.WT) vectors were obtained from PaxVax (San Diego, CA). Wild-type
Ad26 (Ad26.WT) was also tested as a control. The experiments were carried out as
described above in more detail in Example 6 using varying MOIs (100-, 333, 100, 33,
and 10).

[0197] The results are shown in Figure 9 for each cell line tested: A549 (Figure
15 9A), HuTu 80 (Figure 9B), and PER.55K (Figure 9C). The results show that the
rcAd26.Mos1Env vector exhibited a 91-fold reduced replicative capacity as compared
to rcAd4.H5 on day 3 after infection of A549 cells (Figure 9A; $p < 0.0001$), and a 72.5-
fold reduced replicative capacity than rcAd4.H5 on day 3 after infection of HuTu80
cells (Figure 9B; $p = 0.009$). As expected, all vectors tested grew well in PER.55K
20 cells (Figure 9C).

EXAMPLE 10: Mouse Immunogenicity and Infectivity of replicating recombinant rcAd26 vectors.

[0198] The immunogenicity of the following replicating recombinant Ad26
25 vectors according to the invention expressing different mosaic HIV antigens in mice
was assessed: rcAd26.Mos1Env; rcAd26.Mos2Env; rcAd26.Mos1GagPol; and
rcAd26.Mos2GagPol.

[0199] Balb/c mice ($n=4$) were injected intramuscularly (IM) with 10^9 viral
particles (vp) of purified adenovirus rcAd26 vector in PBS ($50 \mu\text{L}$ injection). Mice
30 were sacrificed 28 days after immunization, and the spleen was harvested.
Immunogenicity was determined by assessing the splenocytes by IFN- γ ELISPOT
assay. The splenocytes were stimulated with overlapping peptide pools matching the
mosaic HIV-1 *Gag*, *Pol*, or *Env* sequences in the rcAd26 vectors in a 96-well
multiscreen plate. Each peptide pool consisted of peptides 15 amino acids in length

that overlap by 11 amino acids covering the entire amino acid sequence of the corresponding mosaic antigen. After incubation the wells were washed, labeled and developed to visualize spot forming cells. Cells that responded to the mosaic protein expressed from the injected vector *in vivo* showed up as a spot in the well after *in vitro* stimulation by excreting IFN- γ , which is labeled and visualized using streptavidin-alkaline phosphatase and chromogen.

[0200] The results are expressed as spot forming cells (SFC) per 10^6 splenocytes, and the data is plotted in Figure 10. The results demonstrated that all rcAd26 vectors tested produced immune responses in Balb/c mice to their respective peptide pools.

EXAMPLE 11: Mouse Immunogenicity and Infectivity of Lyophilized rcAd26.Mos1Env.

[0201] The effect of lyophilizing rcAd26.Mos1Env replicating recombinant adenovirus vector according to the invention on mouse immunogenicity and infectivity was tested. Two batches of rcAd26.Mos1Env were lyophilized using different buffers. A sample of each of the lyophilized batches, labeled Lyo#2 and Lyo#3, was reconstituted in phosphate buffered saline and filtered through a 0.22 μ m sterile filter. The virus particle concentration (vp) was determined by spectrophotometry and the vp/PFU results were calculated as shown below in Table 2.

Table 2: vp/PFU Results for rcAd26.Mos1ENV vector

Test Material	Vp/PFU
Non-lyophilized	39
Lyophilized powder – Lyo #2	39
Lyophilized powder – Lyo #3	24

[0202] Balb/c mice (n=4) were immunized by IM injection with 10^8 or 10^9 vp of either reconstituted Lyo #2 or reconstituted Lyo #3 rcAd26.Mos1Env vector. As a control, a non-lyophilized batch of rcAd26.Mos1Env was tested. 28 days after immunization, the mice were sacrificed and the splenocytes were assessed by IFN- γ ELISPOT using overlapping HIV-1 envelope peptide pools matching the HIV-1 Env sequences in the rcAd26.Mos1Env construct, as well as other HIV-1 envelope peptide pools.

[0203] The mosaic peptide pool Mos1Env (split into two subpools: Mos1Env1 and Mos1Env2) consisted of peptides 15 amino acids in length that overlap by 11 amino acids covering the entire amino acid sequence of the mosaic Env protein, Mos1ENV (SEQ ID NO: 48). The potential T-cell epitope (PTE) envelope peptide

pools (HIV PTE Env, split into three subpools: HIV PTE Env1, HIV PTE Env2, HIV
 PTE Env3), obtained from the National Institutes of Health, consisted of peptides of
 15 amino acids in length containing naturally occurring 9 amino acid sequences that
 are potential T cell determinants, captured in an unbiased manner. The PTE peptide
 panel is designed to permit expression of the most frequent potential T cell epitopes
 (PTE) embedded in the sequence of circulating HIV-1 strains of HIV-1 worldwide.
 The IFN- γ ELISPOT assays were performed in the same manner as described above
 in Example 7, and the results are shown in Figure 11A. HIV-1 Clade C envelope and
 Mos1HIVEnv binding antibody ELISA's were performed prior to dosing and 28 days
 after immunization to determine induction of antibodies. The results are shown in
 Figure 11B.

[0204] The results of the ELISPOT and ELISA assays indicate that lyophilized
 and reconstituted rcAd26.Mos1Env remains infectious. Accordingly, the ability of
 the rcAd26.Mos1Env vector to induce an immune response is not affected by
 lyophilization of the vector.

**EXAMPLE 12: Comparison of *In Vivo* Replication of Replication Incompetent
 Ad26 Adenovirus Vector to *In Vivo* Replication of Replication Competent
 rcAd26 Adenovirus Vector in Mice.**

[0205] *In vivo* replication of replication-incompetent Ad26 recombinant
 adenovirus vector (Ad26.Mos1Env) was compared to that of replication-competent
 rcAd26 recombinant adenovirus vector (rcAd26.Mos1Env) in mice by immunizing
 Balb/c mice (n=4) with 10^{10} vp by intramuscular (IM) injection (50 μ l/quadricep) or
 intra-nasally (IN) (25 μ l/nostril). The replication incompetent Ad26.Mos1Env vectors
 contained an E1 deletion (see Figure 1 for schematic diagram of replication
 incompetent recombinant Ad26 vector).

[0206] Serum, rectal and oral swabs were collected from the immunized mice at
 days (d) 0 (wk0), 7 (wk1), 14 (wk2), 21 (wk3) and 28 (wk4). RT-PCR was performed
 on the samples to determine viral shedding. Primers were directed to the hexon
 region of Ad26, in particular the hypervariable regions were targeted to prevent cross
 reactivity with other serotypes. Primers were used in combination with a Taqman
 probe. The primers used were Ad26.RT.fwd 5'- TGCTTACTTTGACGTCCCTG-3'
 (SEQ ID NO: 86) and Ad26.RT.rev 5'- ACTGTTATCTGAAGTTCCTGGC-3' (SEQ
 ID NO: 87). The probe used was Ad26.RT probe 5'-

TTGTATTCTTCCCCACTACCACCTGC-3' (SEQ ID NO: 88). The results of the RT-PCR are shown in Figure 12, and are reported as copies/mL. Control samples were spiked with 5.04×10^6 copies/ml plasmid DNA. The sensitivity of the assay is 1000 copies/mL. The results indicate that there was no evidence of viral shedding.

5 [0207] ELISAs were also performed on the collected serum samples at all time points (days 0, 7, 14, 21, and 28) to determine HIV Clade C Envelope (Figure 13A) and Mosaic Env (Figure 13B) binding antibody titers. The results indicate that both replication-incompetent Ad26.Mos1Env and replication-competent rcAd26.Mos1Env induced HIV-1 Clade C envelope and Mosaic envelope binding antibody titers.

10 [0208] At day 28, the splenocytes were harvested and IFN• ELISPOT was performed to determine immunogenicity in the same manner as described in Examples 6 and 10 (Figure 14). The peptide pools tested for each regimen (i.e., vector and administration route) were Mos1 Env, Mos2 Env, PTE Env 1, PTE Env 2, and PTE Env 3. Results are reported as spot forming cells (SFC) per 10^6 splenocytes.

15 [0209] Although no safety data was collected, it was noted that all of the mice in all groups appeared healthy and well over the course of the study. No adverse effects in mood or physical appearance were observed.

[0210] The results of the ELISA and ELISPOT experiments show that both replication-competent rcAd26.Mos1Env and replication-incompetent Ad26.Mos1Env
20 recombinant adenovirus vectors were immunogenic in mice. The HIV specific antibody responses were similar for the replicating and non-replicating vectors given either by IM or IN administration (Figure 13). The T-cell responses were much greater when the vectors were given by IM administration (Figure 14). RT-PCR results indicated that there was no evidence of replication of vectors in mice, as
25 expected because there is a species barrier for adenoviruses.

EXAMPLE 13: rcAd26.SIVgag Immunogenicity and Replication in Non-Human Primates.

[0211] Non-human primates Indian-origin rhesus monkeys (*Macaca Mulatta*)
30 (N=3/group) were immunized by intramuscular (IM) injection with 10^{11} vp with replication-competent Ad26-SIVGag vector (containing the E1 coding region) and replication-incompetent Ad26-SIVGag vector (lacking the E1 coding region) at weeks 0 and 24. IFN•-ELISPOT was performed at weeks 0, 2, 24, 26, and 32 to determine immune response using a Gag peptide pool (obtained from the National Institutes of

Health) consisting of 15 amino acid peptides overlapping by 11 amino acids, covering the entire Gag sequence. RT-PCR on serum was done at weeks -1, 1, 4, 6, 8, 10, 12 and 16 to look at virus replication as described above in Example 12. The RT-PCR and IFN•-ELISPOT assays were performed as described above in Examples 6 and 10-11, and the results are shown in Figure 15.

[0212] The results demonstrate that both replication-competent and replication-incompetent Ad26.SIVGag vectors are immunogenic in non-human primates. There was no evidence by RT-PCR that the replication-competent vector replicates in non-human primates. As with the experiments described in Example 12 above in mice, and demonstrated by Example 7 (Figure 7), it was expected that the adenovirus vectors would not replicate in non-human primates due to a species barrier.

EXAMPLE 14: Cloning of Ad35 Adaptor plasmid lacking a transgene (pAdApt35BSU.E1atg.Empty) with E1 coding region after transgene cassette.

[0213] The empty Ad35 recombinant vector, pAdApt35BSU.E1atg.Empty was constructed as shown in Figure 16A. The pAdApt35BSU.E1atg.Empty vector contains the E1 coding region after the transgene cassette. “Empty” denotes that the vector does not contain a heterologous nucleic acid sequence or transgene, however any heterologous nucleic acid sequence of interest can be inserted into the multiple cloning site of the transgene cassette of the pAdApt35BSU.E1atg.Empty vector under control of the CMV promoter and upstream of the E1 coding region. For example, a nucleic acid sequence encoding the mosaic antigen Mos1-HIVEnv can be cloned into the multiple cloning site of the transgene cassette of pAdApt35BSU.E1atg.Empty to obtain the Ad35 recombinant vector pAdApt35BSU.E1atg. Mos1-HIVEnv (SEQ ID NO: 84), with the Mos1-HIVEnv gene located before the E1 coding region.

Production of “PCR Fragment A” and “PCR Fragment B”

[0214] Two PCRs were performed to clone the E1 region after the transgene cassette. The first PCR was to amplify the transgene cassette from the pAdApt35BSU, and was performed using a forward primer that overlaps with the existing NdeI site in the CMV promoter and a reverse primer designed to contain a MluI site that overlaps with the polyA region. The forward primer used was AdApt35BSU.NdeI.fwd having the sequence 5’-

GTGTATCATATGCCAAGTACGCCC-3' (SEQ ID NO: 89), and the reverse primer used was AdApt35BSU.MluI.rev having the sequence 5-

CGATCACGCGTATCTAGACATGATAAGATACATTGATG-3' (SEQ ID NO: 90). PCR fragment A was obtained from this reaction. See the schematic labeled

5 "pAdApt35BSU PCR Fragment A" of Figure 16A.

[0215] The second PCR was to amplify the E1 coding region from the wild-type adenovirus vector genome ("Ad35 WT"), and was performed using a forward primer designed to contain a MluI site that starts at nucleotide 464 of the Ad35 WT sequence. The reverse primer was designed to overlap with the existing AleI site in pIX region.

10 . The forward primer used was Ad35WT.464.MluI.fwd having the sequence 5'-CACAGACGCGTCTGATCGCTAGGGTATTTATACCTC-3' (SEQ ID NO: 91), and the reverse primer used was Ad35WT.AleI.rev having the sequence 5'-GGAGGACACAAGGGTGTCTCCAAA-3' (SEQ ID NO: 92). PCR fragment B was obtained from this reaction. See the schematic labeled "Ad35 WT PCR Fragment
15 B" of Figure 16A.

Cloning of pAdapt35BSU.E1atg.Empty (E1 region after transgene cassette)

[0216] PCR fragment A was digested with NdeI and MluI. PCR fragment B was digested with MluI and AleI. pAdApt35BSU.Empty was digested with NdeI and AleI.
20 Digestions were gel purified and ligated together in a triple ligation resulting in pAdApt35BSU.E1atg.Empty.

EXAMPLE 15: Cloning of Ad35 Adaptor plasmid lacking a transgene (pAdApt35BSU.Empty) with E1 coding region before transgene cassette.

25 [0217] The empty Ad35 recombinant vector, pAdApt35BSU.E1btg.Empty (SEQ ID NO: 26) was constructed as shown in Figure 16B. The pAdApt35BSU.E1btg.Empty vector contains the E1 coding region before the transgene cassette. "Empty" denotes that the vector does not contain a heterologous nucleic acid sequence or transgene, however any heterologous nucleic acid sequence
30 of interest can be inserted into the multiple cloning site of the transgene cassette of the pAdApt35BSU.E1btg.Empty vector under control of the CMV promoter and downstream of the E1 coding region. For example, a nucleic acid sequence encoding the mosaic antigen Mos1-HIVEnv can be cloned into the multiple cloning site of the transgene cassette of pAdApt35BSU.E1btg.Empty to obtain the Ad35 recombinant

vector pAdApt35BSU.E1btg. Mos1-HIVEnv (SEQ ID NO: 85), with the Mos1-HIVEnv gene located after the E1 coding region.

Mutation of PacI site in E1 gene

5 [0218] The Ad35 Wild Type E1 coding region contains a PacI restriction enzyme site. To inactivate the PacI site, site-directed mutagenesis was performed. The PacI site was inactivated by changing one base pair from a T to a C using rcAd35.E1mut.fwd 5'-GTTTTATTTTAATCAAGGGAAATGCCA-3' (SEQ ID NO: 64) as forward primer, rcAd35.E1mut.rev 5'-

10 TGGCATTTCCTTGATTAAAATAAAAC-3' (SEQ ID NO: 65) was used as reverse primer, and pAdApt35BSU.E1.atg.Empty as template (obtained from Example 14) in a PCR covering the entire plasmid. After completion of the PCR, the PCR mix was digested with DpnI enzyme to remove the

15 pAdApt35BSU.E1.atg.Empty template DNA. A small aliquot of the digested PCR mix was transformed into *E. coli* and clones containing the mutated PacI site were screened by restriction enzyme analysis and sequencing.

Cloning of pAdApt35BSU.E1btg.Empty (E1 region before transgene cassette)

[0219] To clone the E1 region before the transgene cassette, a PCR was

20 performed using pAdApt35BSU.E1atg.Empty as template. The forward primer used was located at the start of the E1 region right after the polyA sequence (nt464 in Ad35 WT), and the reverse primer is located at the end of the E1 region before the pIX (nt3483 in Ad35 WT). Both forward and reverse primer had AvrII sites designed in them. The sequences of the primers were as follows: 35BSU.E1btg.AvrII.fwd 5'-

25 ATAACACCTAGGCTCATCGCTAGGGTATTTATACCTC-3' (SEQ ID NO: 82) and 35BSU.E1btg.AvrII.rev 5'-

ATAACACCTAGGTTAGTCAGTTTCTTCTCCACTGGAT-3' (SEQ ID NO: 83).

[0220] After digesting both the PCR product as well as pAdApt35BSU with AvrII, the PCR product and pAdApt35BSU were ligated together resulting in

30 pAdApt35BSU.E1btg.Empty that also contained the mutated PacI site in the E1 coding region. Screening of the clones was done by restriction enzyme analysis and sequencing.

EXAMPLE 16: Cloning of Ad35 cosmid vector (pWe/Ad35.pIX-rITR.dE3.dE3.35orf6).

Cloning of full cosmid pWe/Ad35.pIX-rITR.dE3.dE3.35orf6 vector (SEQ ID NO: 25).

5 [0221] A PCR was done to obtain the pWe cosmid backbone with a NotI site on each end using the following primer pair: forward primer, pWe.NotI.fwd 5'-AATTTAGCGGCCGCATCGTCCATTCCGACAGCATCGC-3' (SEQ ID NO: 66); and reverse primer, pWe.NotI.rev 5'-GAAGCATTTCCACTCATGTCTG (SEQ ID NO: 67). The 5' NotI site was designed in the primer. Both the pWe/Ad35.pIX-EcoRV and pBr/Ad35.dE3.dE4.35orf6 plasmids were digested with NotI and RsrII and gel purified (see Vogels et al., Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of preexisting adenovirus immunity *J. Virology* (2003) 77(15) 8263-71 for a description of both plasmids). A three point ligation was done to obtain the full length

10 pWe/Ad35.pIX-rITR.dE3.dE4.35orf6 cosmid vector (SEQ ID NO: 25), which was screened by digestions and sequencing.

[0222] In this Example, E1 was cloned both before and after the transgene cassette. It was observed that for the rcAd35 vectors, when E1 is placed downstream of the transgene cassette, there was some instability of the rcAd35 vector, while when

20 E1 is placed upstream of the transgene cassette, this instability was not seen.

[0223] Accordingly, in a preferred embodiment of the invention, the heterologous nucleic acid sequence is located between the left ITR and the 5'-end of the functional E1 coding region, i.e., the functional E1 coding region is placed upstream of the heterologous nucleic acid sequence.

25

EXAMPLE 17: Clinical Study of the Safety and Immunogenicity of Oral Replicating Recombinant Ad26 Vector Vaccine for HIV-1 in Uninfected Human Adults.

[0224] Replicating recombinant rcAd26.MOS1.HIVEnv vaccine vector is tested

30 for safety and immunogenicity against HIV-1 in adult humans uninfected with HIV-1 when administered as a single oral dose in a randomized, controlled, double-blind phase 1 clinical trial. Objectives of the study include evaluating the safety and tolerability of the vaccine, determining viral shedding in rectal and oropharyngeal

secretions, and evaluating humoral and cellular immune responses elicited by four increasing dosages of the vaccine vector.

[0225] The study population includes healthy men and women aged 18-40 years old. The vaccine is formulated as an enteric-coated capsule for oral administration.

5 Study participants are divided into four groups, with each receiving a different dosage of the rcAd26.MOS1.HIVEnv vaccine: 10^8 viral particles (“vp”)/placebo capsule, 10^9 vp/placebo capsule, 10^{10} vp/placebo capsule, or 10^{11} vp/placebo capsule. Study participants receiving placebo are administered a sucrose-containing capsule. Since the vaccine is replication competent, study participants are housed in an isolation unit
10 beginning two days prior to administration of the vaccine, and continuing until at least nine days post-vaccination. The study lasts for approximately twelve months.

[0226] Rectal and throat swabs are obtained from the study participants to evaluate viral shedding. In particular, the rectal and throat swabs are analyzed for the presence of rcAd26.MOS1.HIVEnv by PCR and adenovirus culture. Real-time (rt)-
15 PCR is performed for both the Ad26 vector and Env insert to allow for quantification of vector and insert, and to evaluate the stability of the vaccine. Adenovirus culture is performed to assess the presence of infectious virus. Serotyping is further be performed on positive cultures to confirm that the virus is rcAd26.MOS1.HIVEnv.

[0227] Blood samples are obtained from each of the study participants at various
20 time points after immunization to assess HIV-associated immunogenicity, including assessment of cellular immune and humoral immune response. Blood samples are assayed for the magnitude of antibody binding to HIV-1 Env as quantified by enzyme-linked immunosorbent assay (ELISA), the magnitude of neutralizing antibodies against HIV-1 as quantified by virus neutralization assay, and the
25 magnitude of HIV-1 specific T-lymphocyte responses as quantified by IFN- γ ELISPOT. Blood samples can also be used for exploratory DNA and RNA micro-array and deep-sequencing assays.

[0228] Additional immunogenicity assessments of systemic and mucosal responses can include HIV-1 specific antibody-dependent cell-mediated cytotoxicity
30 (ADCC) and cell-mediated viral inhibition (ADCVI) assays, measurement of Ad26-specific antibodies (e.g., ADCC, ADCVI, etc.), intracellular cytokine staining (ICS) assays, flow cytometry of CD4+ and CD8+ T cell-mediated inhibition of HIV-1, proliferative capacity of T lymphocytes, B cell repertoire analysis, gene expression profiling, and epitope mapping.

[0229] Additionally, rectal mucosal secretions are collected from the study participants with rectal wicks for evaluation of potential mucosal immunity to HIV-1 Env and Ad26. Such studies can include measurement of Ad26 and HIV-1 specific antibodies by ELISA, and antibody epitope mapping to Env peptides by linear peptide
5 microarray.

[0230] The possibility of transmission of the rcAd26.MOS1.HIVEnv to volunteers' household contacts, i.e., an individual who shares the same residence as the study participant, is evaluated. Household contacts of the study participants are contacted on a weekly basis to assess for the presence of illness that may be related to
10 transmission of the vaccine vector.

[0231] Throughout the course of the study, participants are also monitored for the occurrence of any adverse events.

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It will be appreciated by those skilled in the art that changes could be made to the embodiments described above without departing from the broad inventive concept thereof. It is understood, therefore, that this invention is not limited to the particular embodiments disclosed, but it is intended to cover modifications within the spirit and
5 scope of the invention as defined by the appended claims.

CLAIMS

We claim:

- 5 1. A replicating recombinant adenovirus vector, comprising a recombinant adenovirus genome having:
 - (a) a promoter operably linked to a heterologous nucleic acid sequence;
 - (b) a functional E1 coding region;
 - (c) a deletion in the E3 coding region; and
 - (d) a deletion in the E4 coding region, provided that E4 open reading frame
- 10 6/7 is not deleted,
wherein the adenovirus genome is human adenovirus serotype 26 or 35 genome.
- 15 2. The replicating recombinant adenovirus vector of claim 1, wherein the heterologous nucleic acid sequence is located between left ITR and 5'-end of the functional E1 coding region
3. The replicating recombinant adenovirus vector of any one of claims 1 and 2, wherein the heterologous nucleic acid sequence encodes an immunogenic polypeptide.
- 20 4. The replicating recombinant adenovirus vector of any of the preceding claims 1 to 3, wherein the heterologous nucleic acid sequence encodes an HIV antigen.
5. The replicating recombinant adenovirus vector of any of the preceding claims 1 to 4, wherein the heterologous nucleic acid sequence encodes a mosaic HIV antigen.
- 25 6. The replicating recombinant adenovirus vector of any of the preceding claims 1 to 5, wherein the heterologous nucleic acid sequence encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 48 or SEQ ID NO: 50
7. The replicating recombinant adenovirus vector of any of the preceding claims 1 to 6, wherein the vector is lyophilized.

8. The replicating recombinant adenovirus vector of any of the preceding claims 1 to 7, wherein the replicative capacity of the vector is attenuated by at least about 80-fold, as compared to the replicative capacity of an wild-type human adenovirus serotype 26 or 35, respectively.
- 5 9. A replicating recombinant adenovirus vector comprising a recombinant human adenovirus serotype 26 genome having:
- (a) a promoter operably linked to a heterologous nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 48 or SEQ ID NO: 50;
- (b) a functional E1 coding region encoding the amino acid sequences of SEQ
10 ID NOs: 14-16;
- (c) a partially deleted E3 coding region consisting of the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 6; and
- (d) a partially deleted E4 coding region consisting of the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 22.
- 15 10. The replicating recombinant adenovirus vector of claim 9, wherein the heterologous nucleic acid sequence is located between left ITR and 5'-end of the functional E1 coding region.
11. The replicating recombinant adenovirus vector of any of the preceding claims 9-10, wherein,
- 20 a. the promoter is CMV promoter having the nucleotide sequence of SEQ ID NO: 51, and the heterologous nucleic acid sequence comprises the nucleotide sequence of SEQ ID NO: 47 or SEQ ID NO: 49;
- b. the functional E1 coding region comprises the nucleotide sequence of SEQ ID NO: 13;
- 25 c. the partially deleted E3 coding region consists of the nucleotide sequence of SEQ ID NO: 5; and
- d. the partially deleted E4 coding region consists of the nucleotide sequence of SEQ ID NO: 23.
12. A composition comprising the replicating recombinant adenovirus vector of
30 any of the preceding claims 1-11 and a pharmaceutically acceptable carrier.

13. The composition of claim 12 being formulated for oral administration to a subject.

14. The composition of any one of claims 12 or 13, being an enteric-coated capsule.

5 15. A method of producing a replicating adenovirus particle, comprising:

introducing the replicating recombinant adenovirus vector of any one of claims 1-11 into a cell under conditions sufficient for replication of the recombinant adenovirus genome and packaging of the adenovirus particle in the cell; and

10 collecting the adenovirus particle.

16. A method of producing an immune response in a subject, comprising:

administering to the subject an immunogenically effective amount of the composition according to any one of claims 12-14.

17. The method of claim 16, wherein the composition is orally administered to the subject.

18. A method of vaccinating a subject against an infection, comprising:

administering to the subject an immunogenically effective amount of a composition according to any one of claims 12-14.

19. A method of vaccinating a human subject against an HIV infection,

20 comprising: orally administering to the subject an immunogenically effective amount of a composition comprising a pharmaceutically acceptable carrier and a replicating recombinant adenovirus vector comprising a recombinant serotype 26 adenovirus genome having:

25 (a) a promoter operably linked to a heterologous nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 48 or SEQ ID NO: 50;

(b) a functional E1 coding region encoding the amino acid sequences of SEQ ID NOs: 14-16;

(c) a partially deleted E3 coding region consisting of the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 6; and

(d) a partially deleted E4 coding region consisting of the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 22,

wherein the heterologous nucleic acid sequence is located between left ITR and 5'-end of the functional E1 coding region.

5 20. The method of claim 19, wherein

a. the promoter is CMV promoter having the nucleotide sequence of SEQ ID NO: 51 and the heterologous nucleic acid sequence comprises the nucleotide sequence of SEQ ID NO: 47 or SEQ ID NO: 49;

10 b. the functional E1 coding region comprises the nucleotide sequence of SEQ ID NO: 13;

c. the partially deleted E3 coding region consists of the nucleotide sequence of SEQ ID NO: 5; and

d. the partially deleted E4 coding region consists of the nucleotide sequence of SEQ ID NO: 23.

15 21. A composition of any one of claims 12 to 14 for use in producing an immune response in a subject or vaccinating a human subject against an infection, preferably, for use in vaccinating a human subject against an HIV infection.

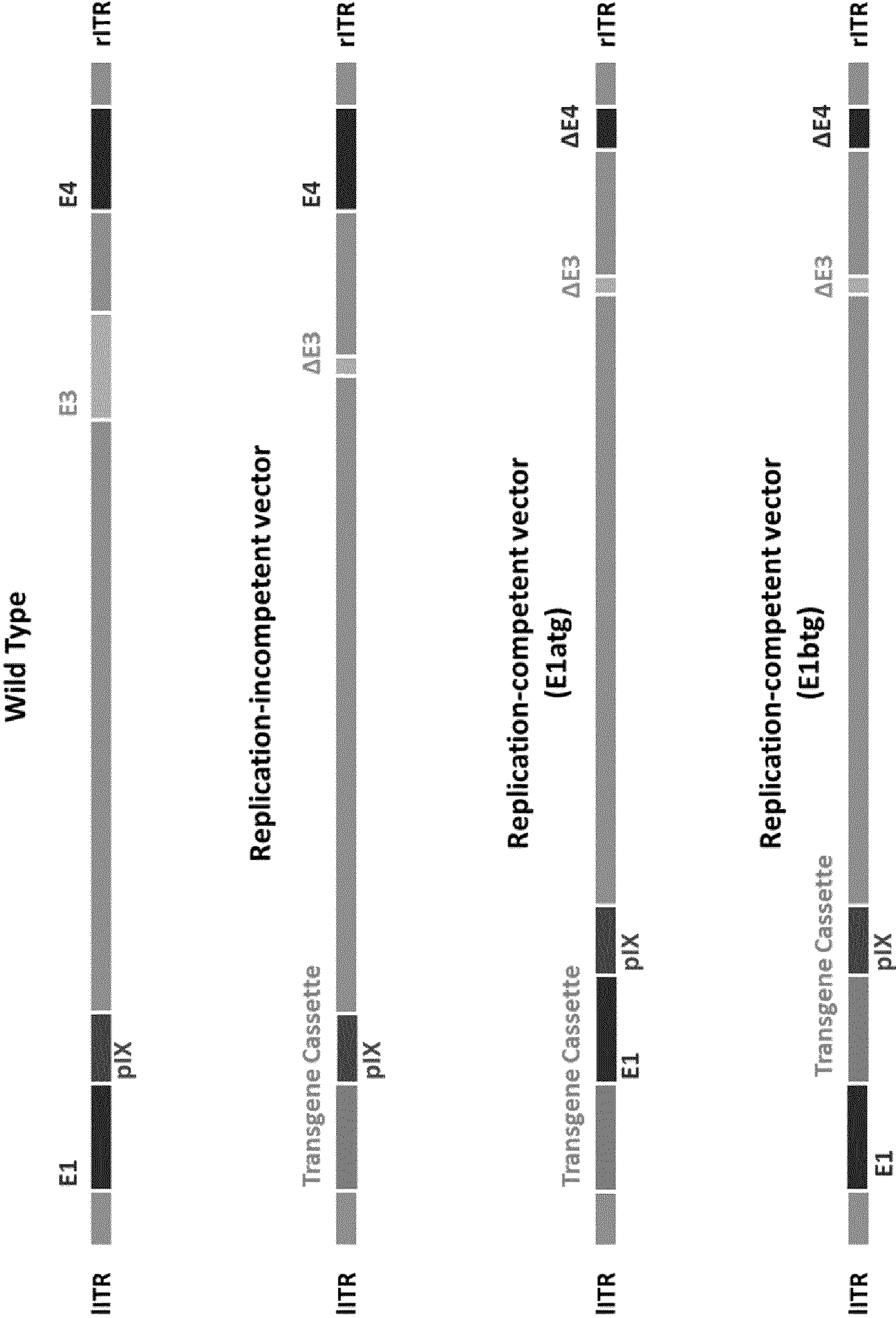


Fig. 1

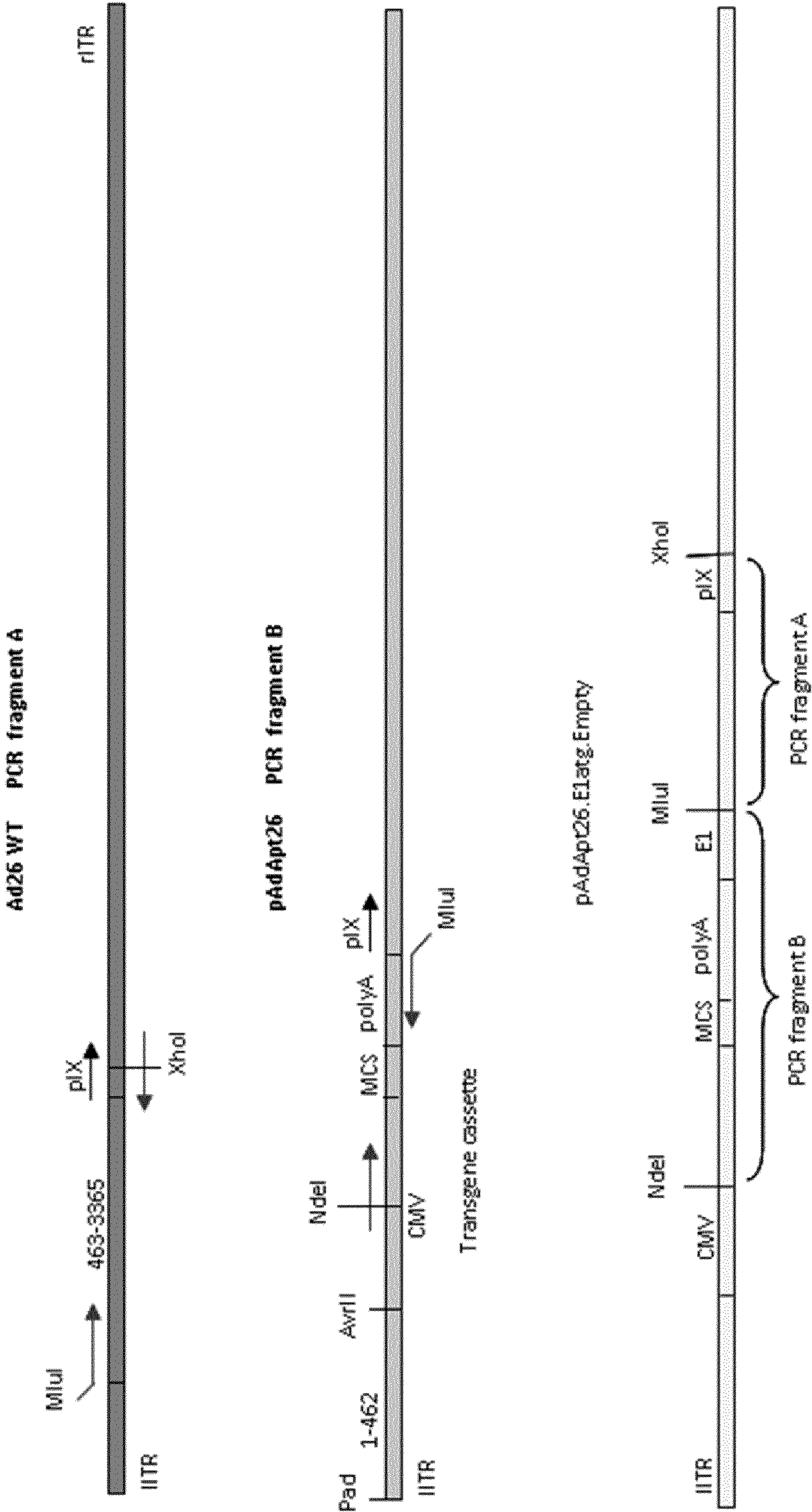


Fig. 2

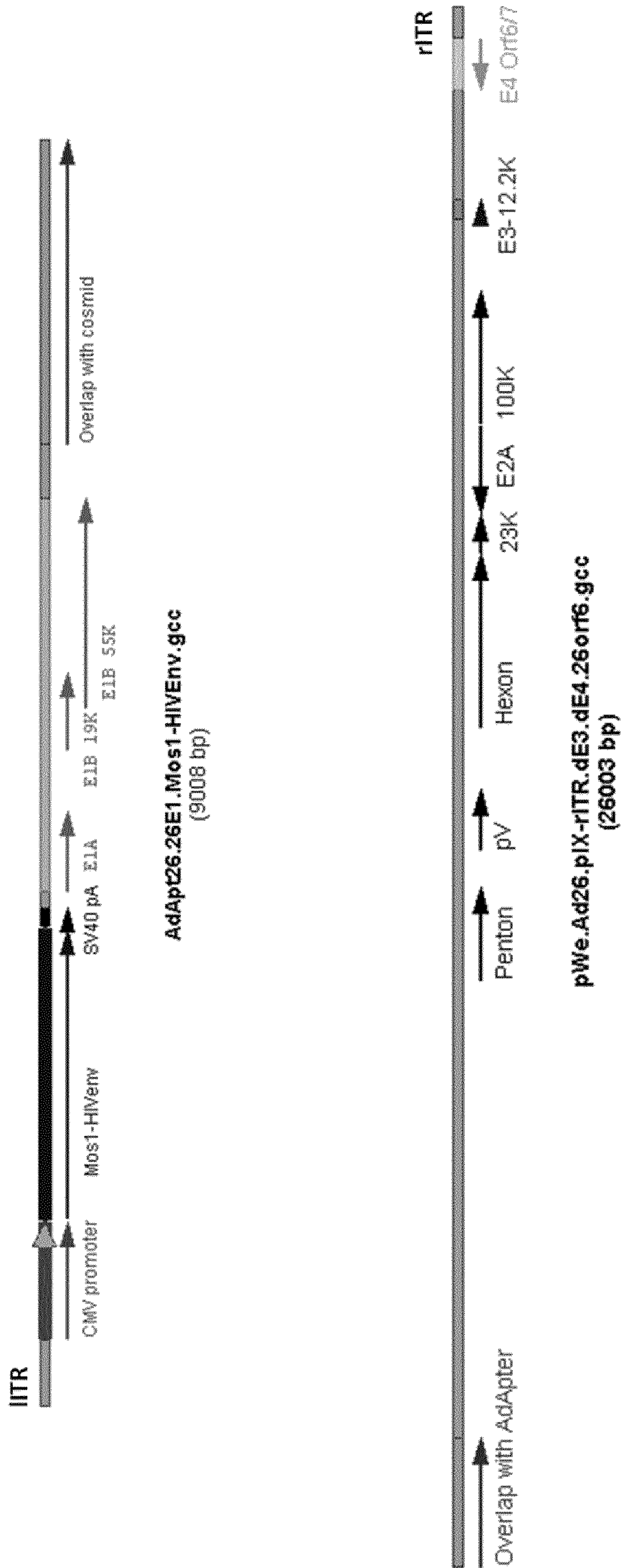


Fig. 3

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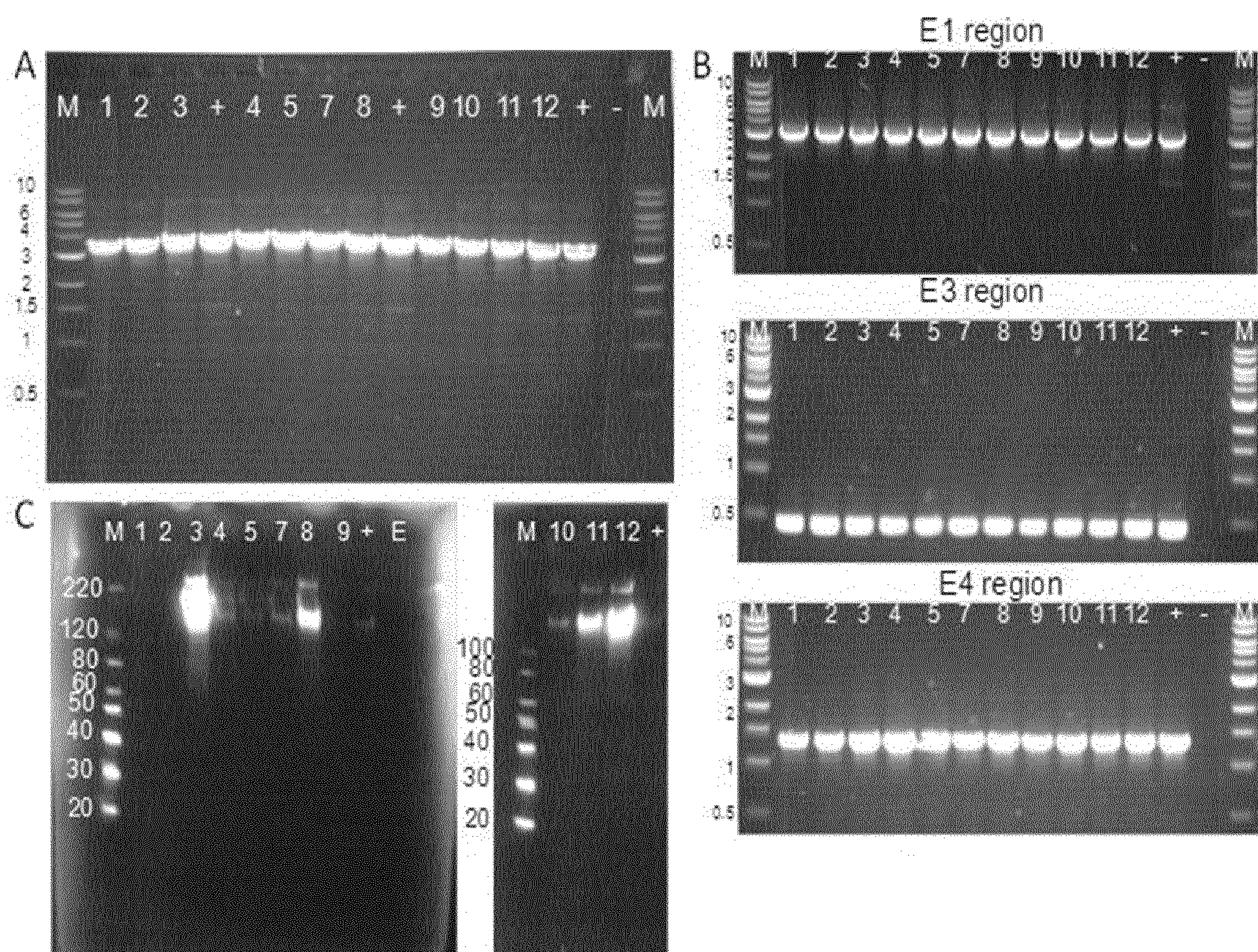


Fig. 4

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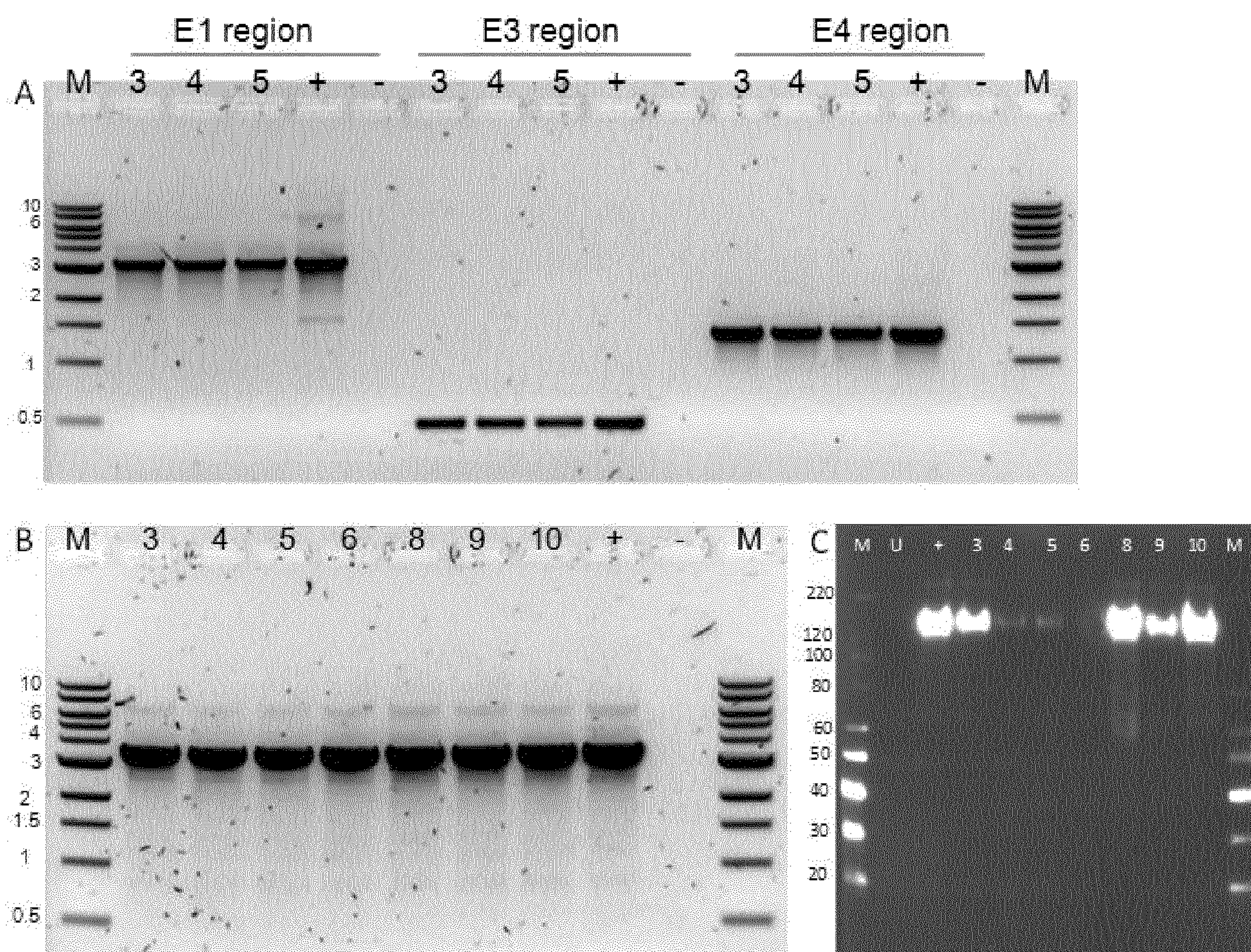


Fig. 5

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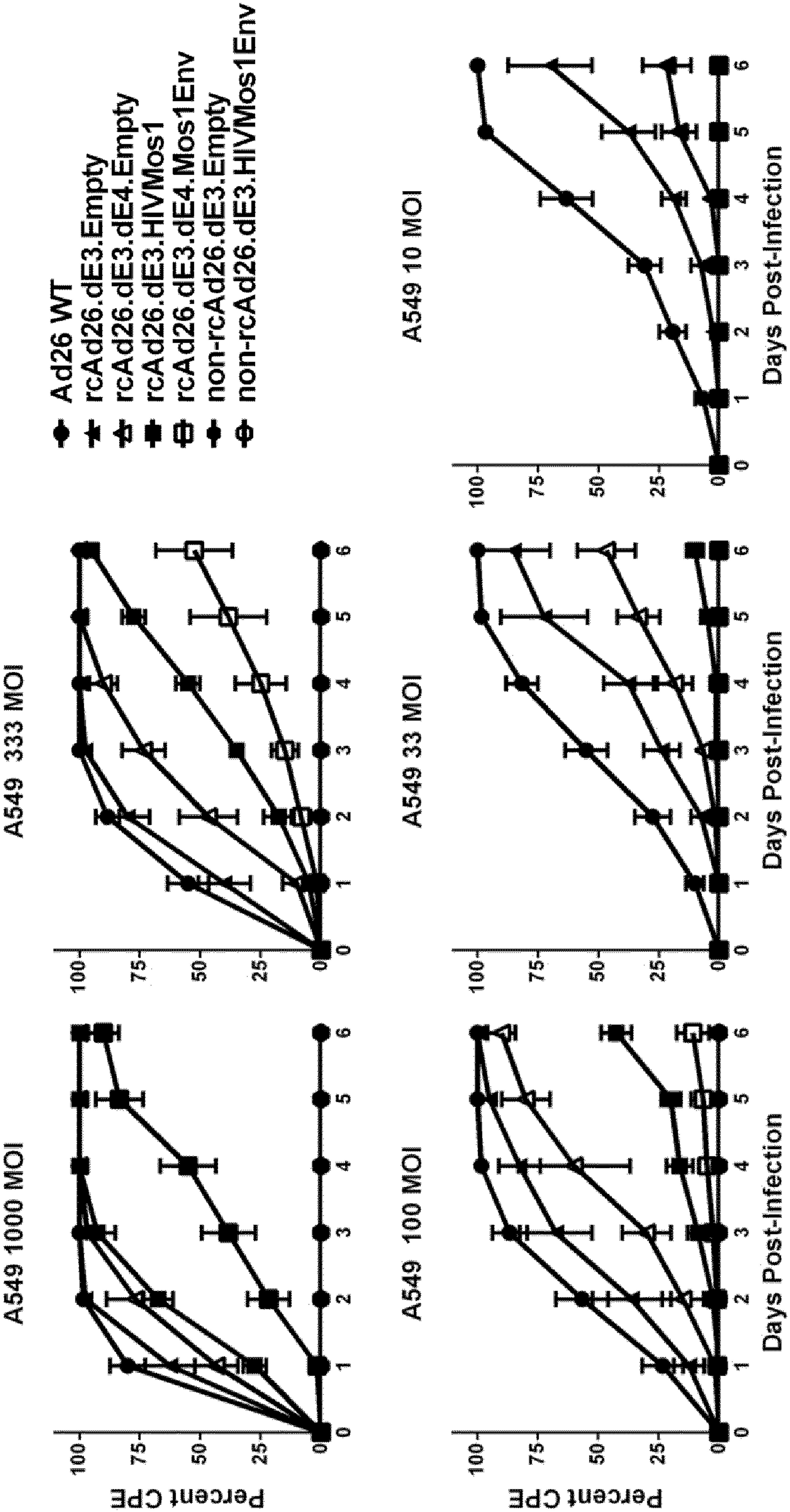


Fig. 6 A

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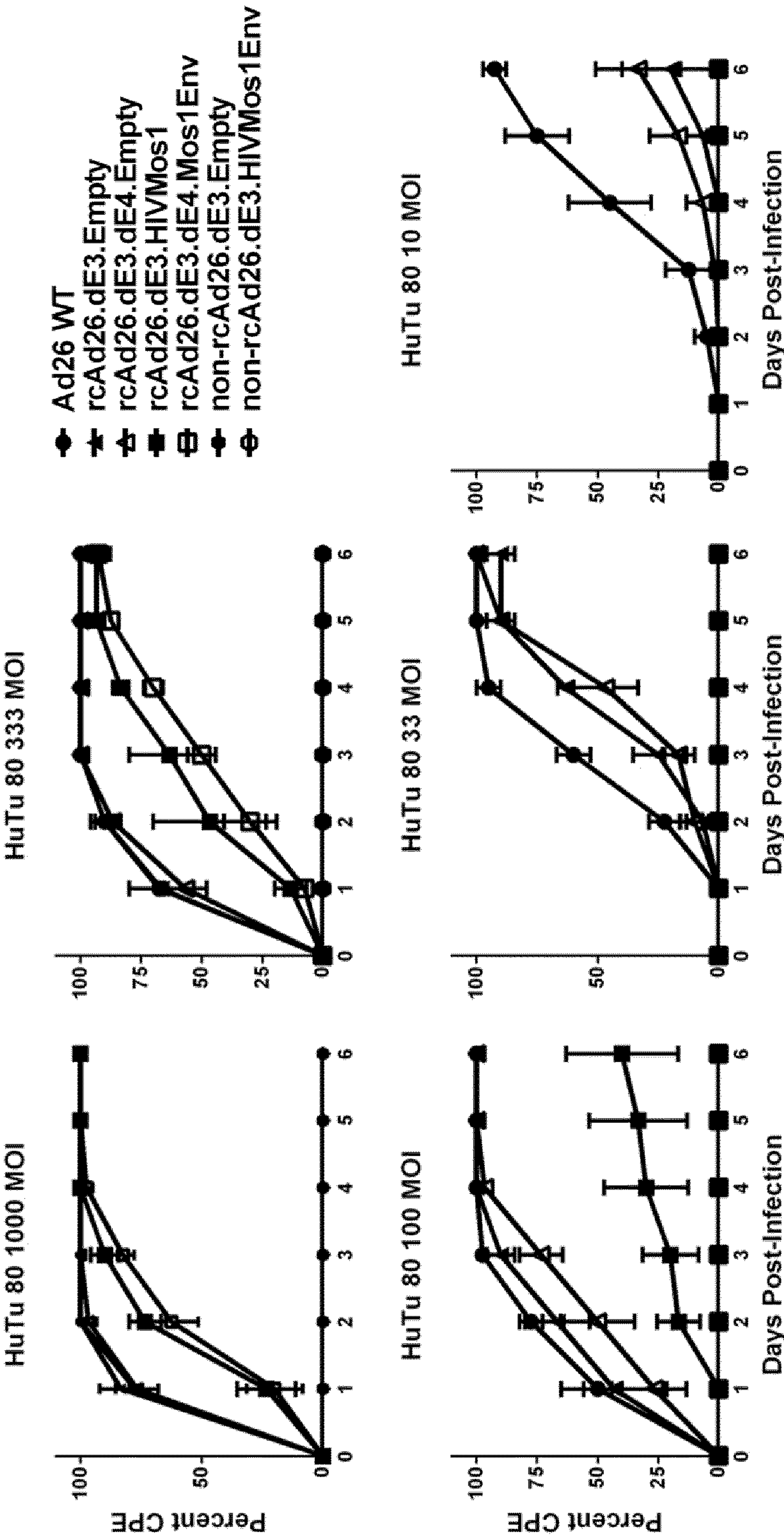


Fig. 6 B

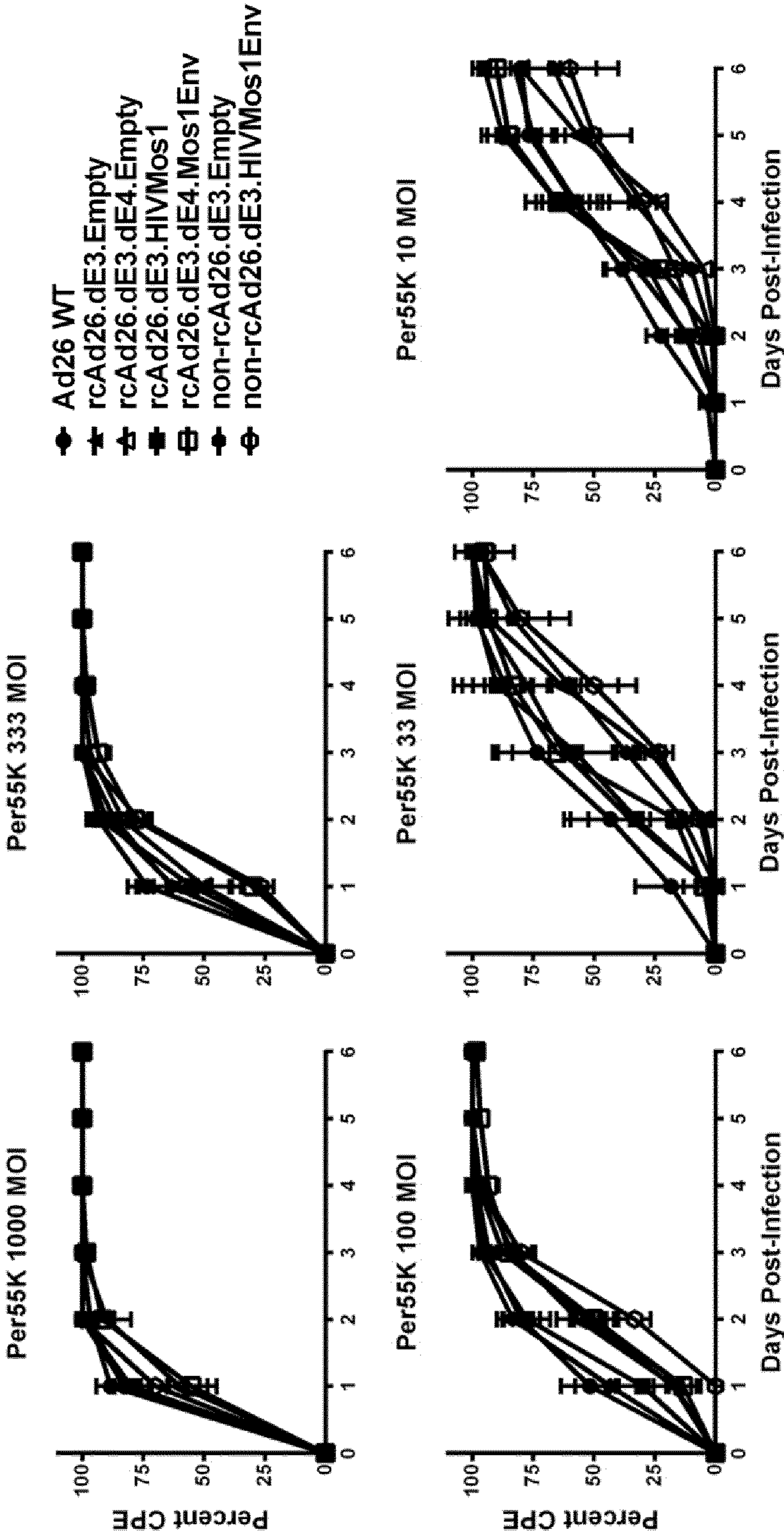


Fig. 6 C

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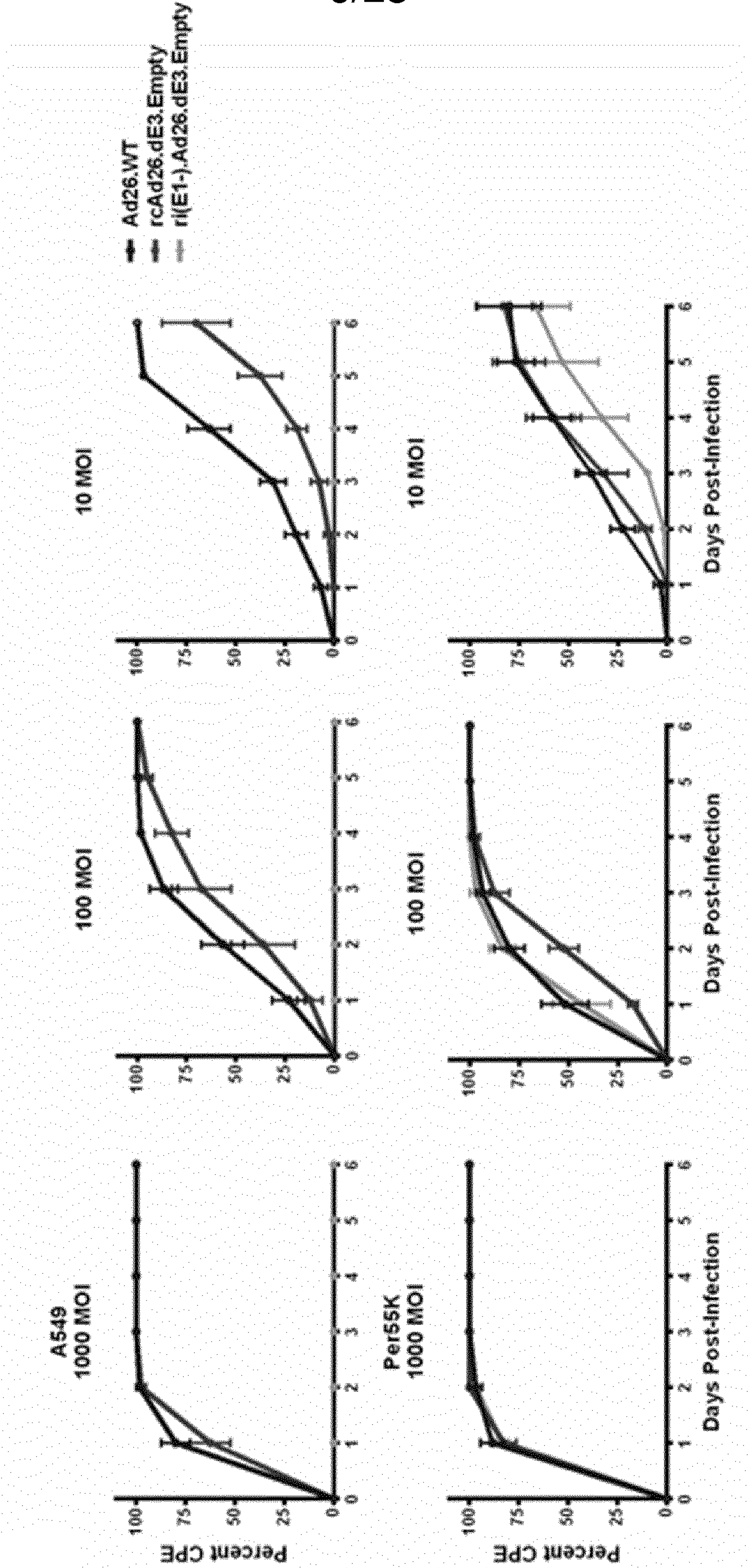


Fig. 7 A

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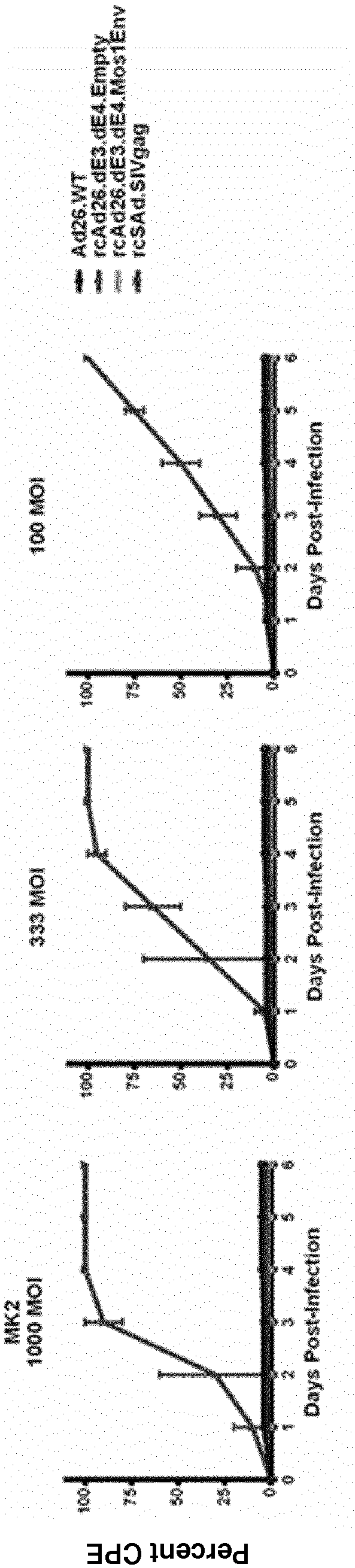


Fig. 7 B

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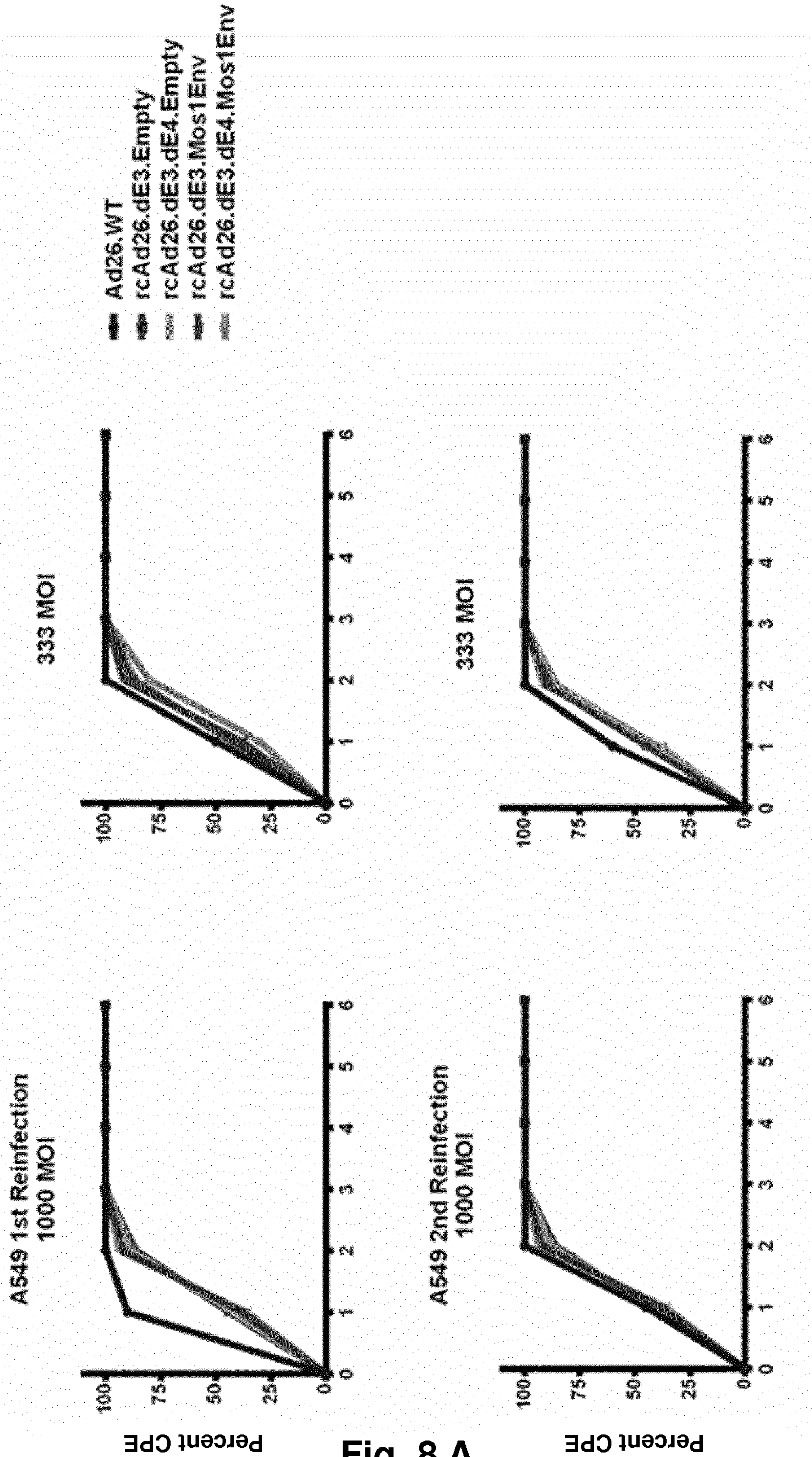


Fig. 8 A

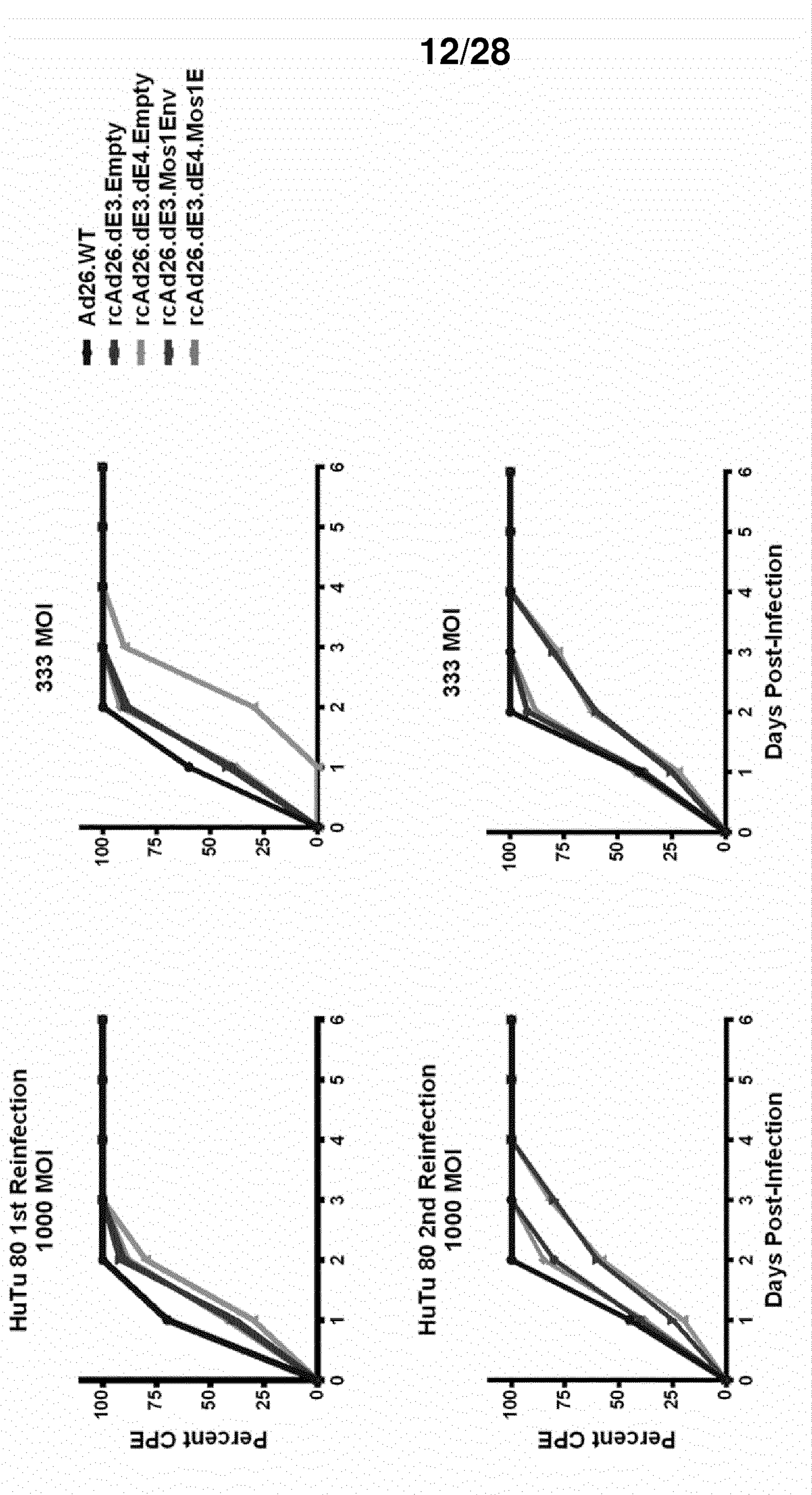


Fig. 8 B

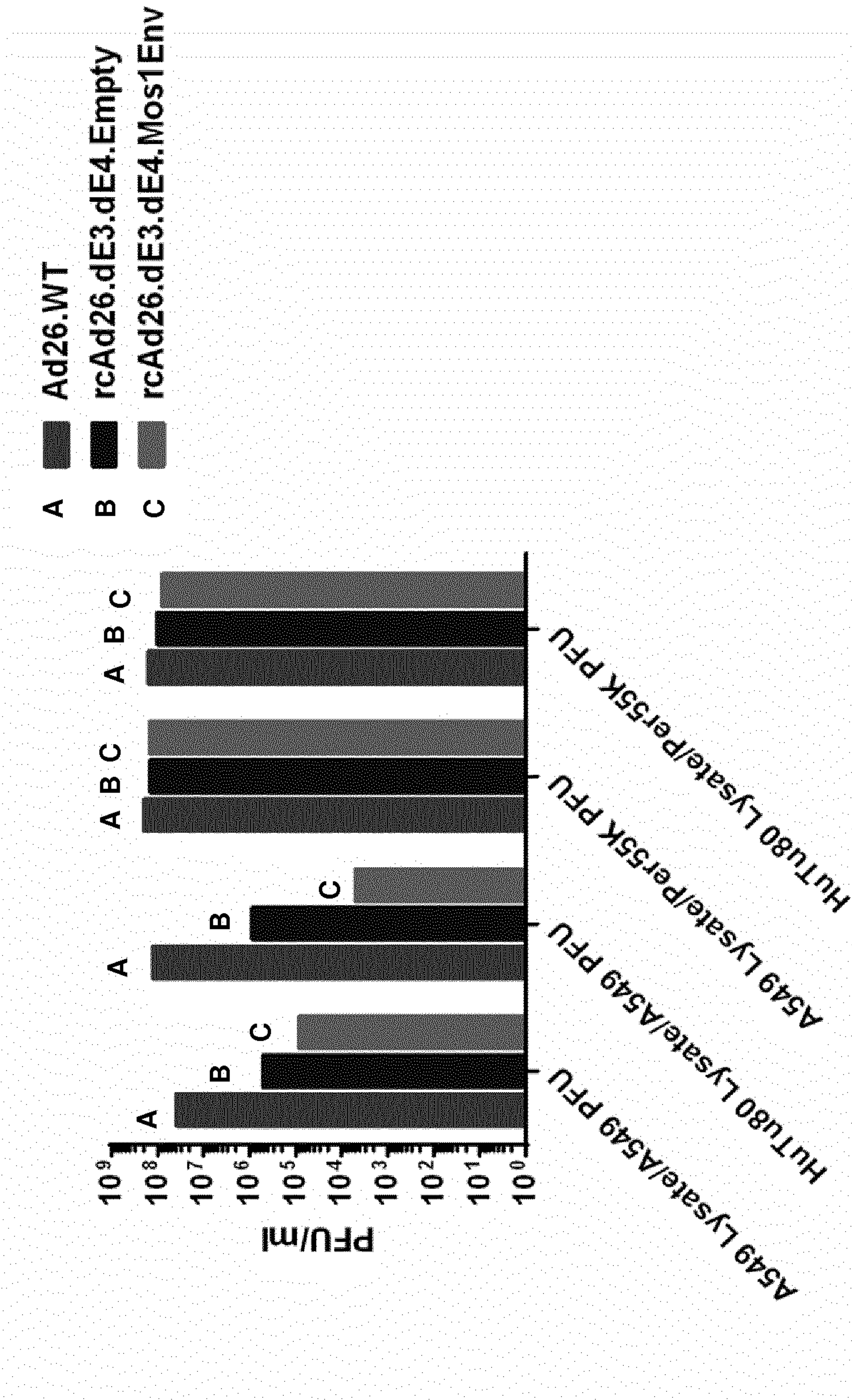


Fig. 8 C

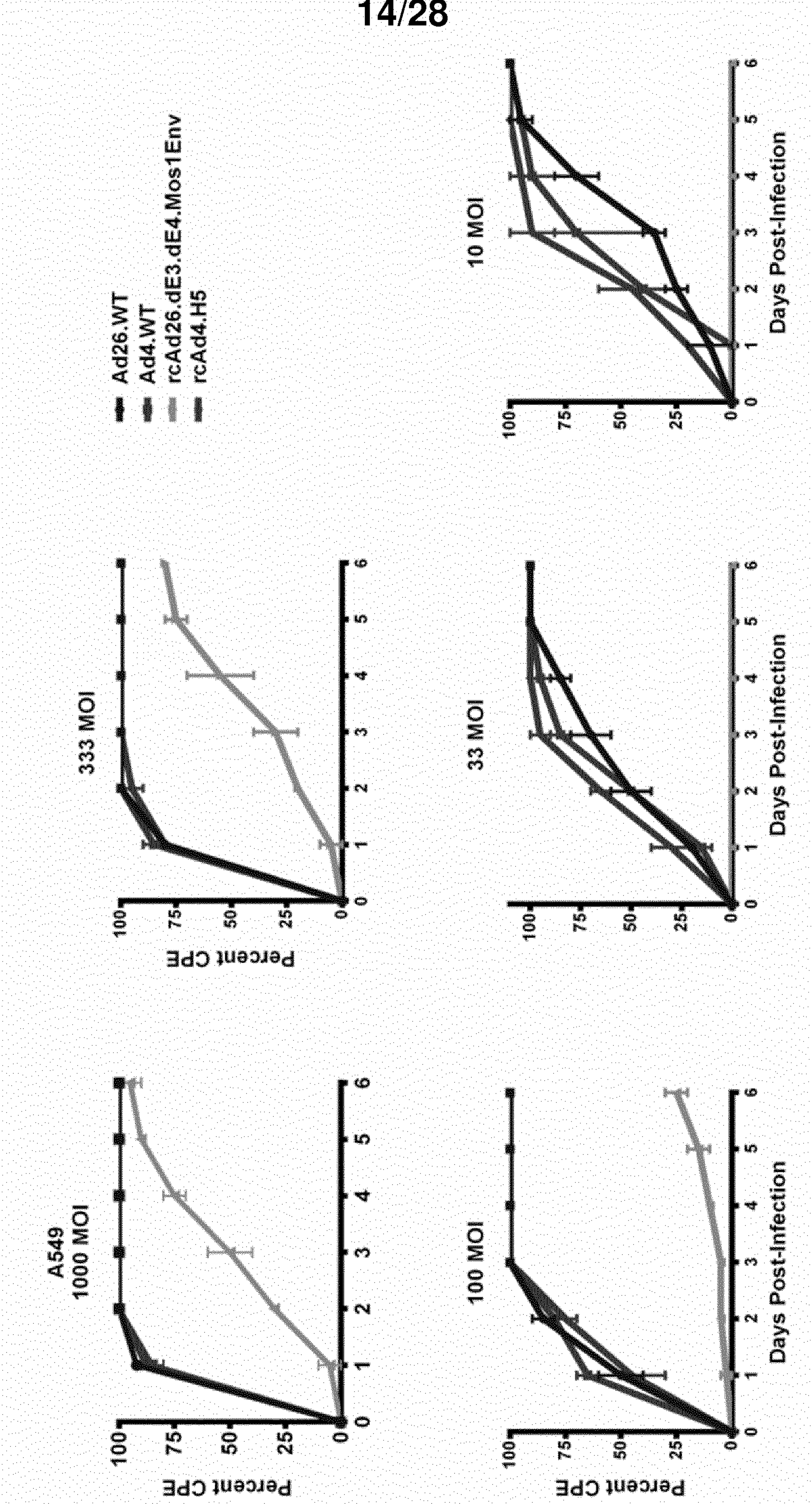


Fig. 9 A

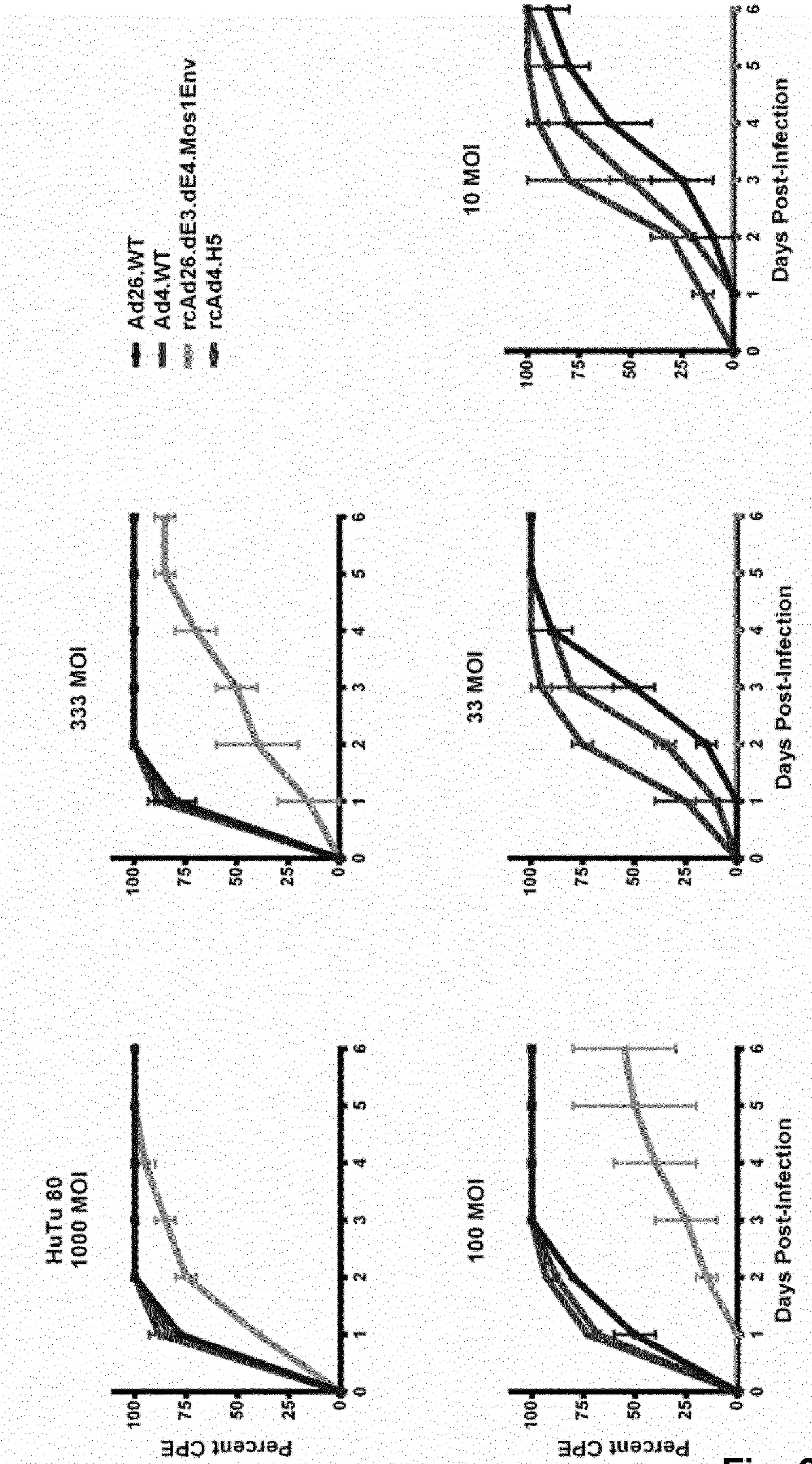


Fig. 9 B

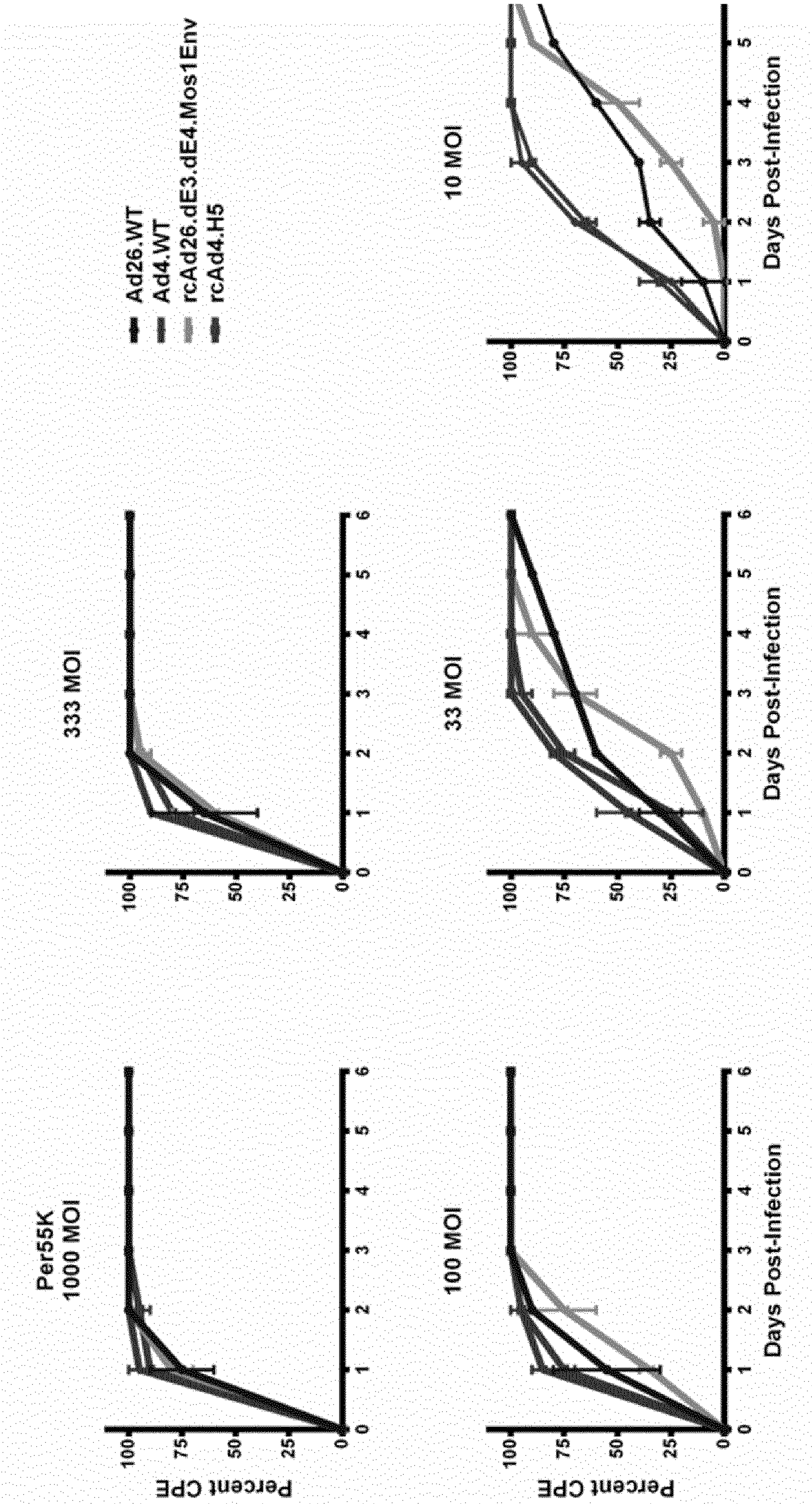


Fig. 9 C

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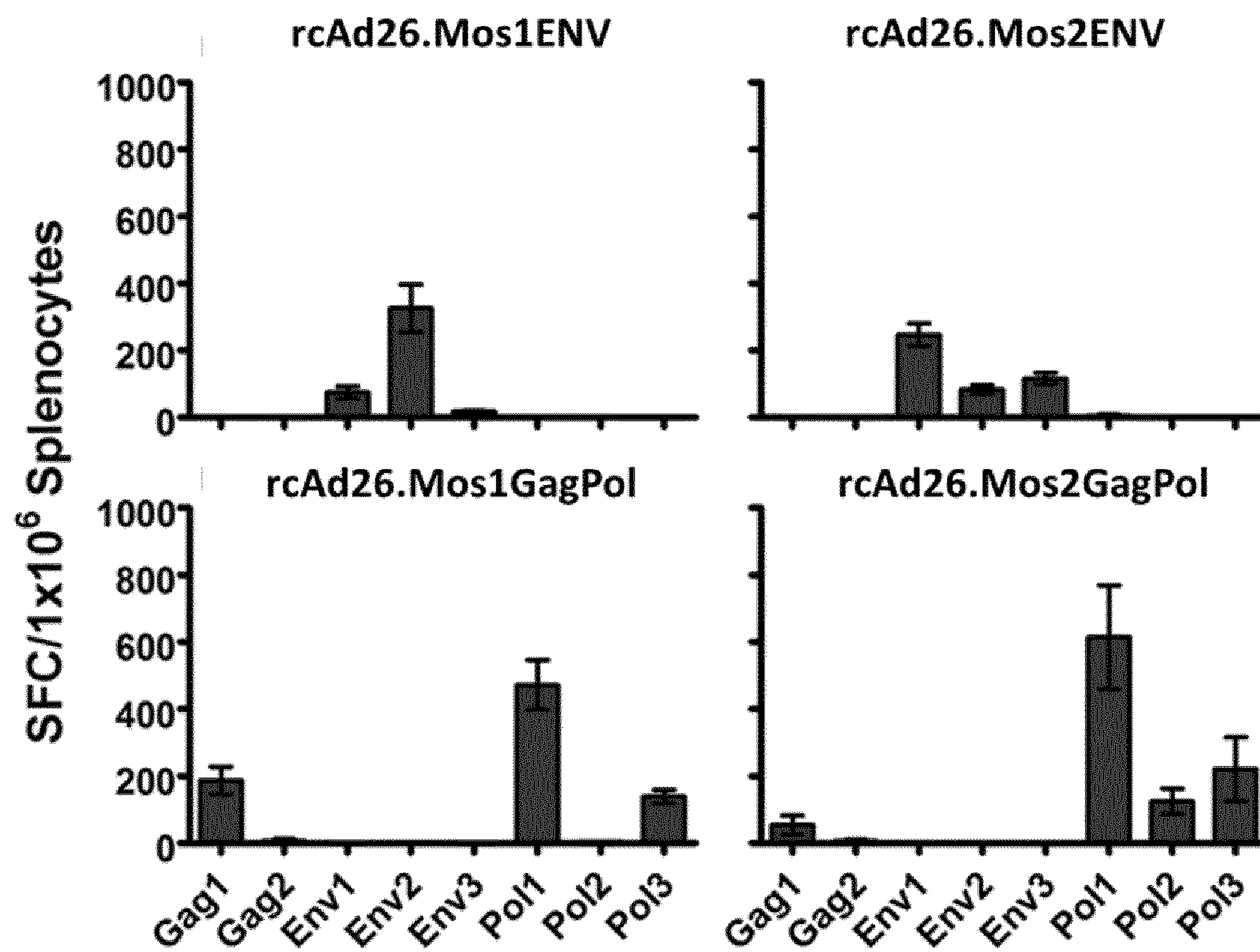


Fig. 10

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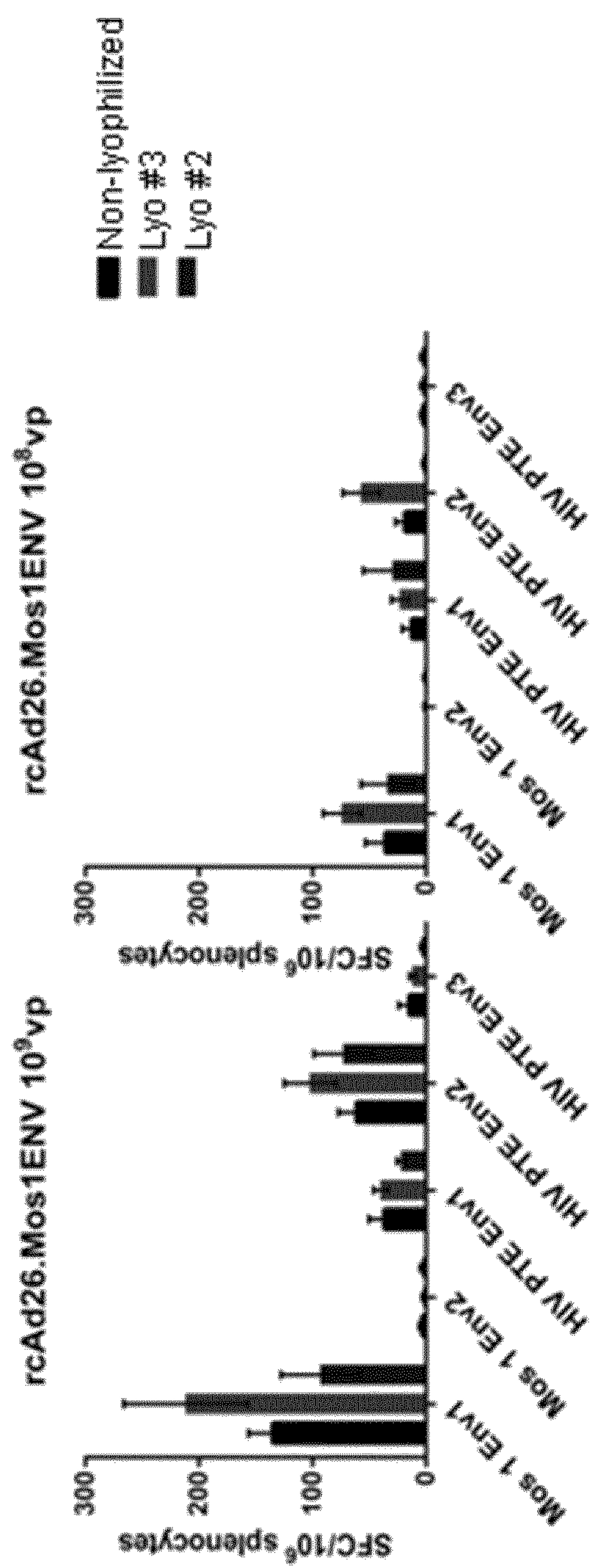


Fig. 11 A

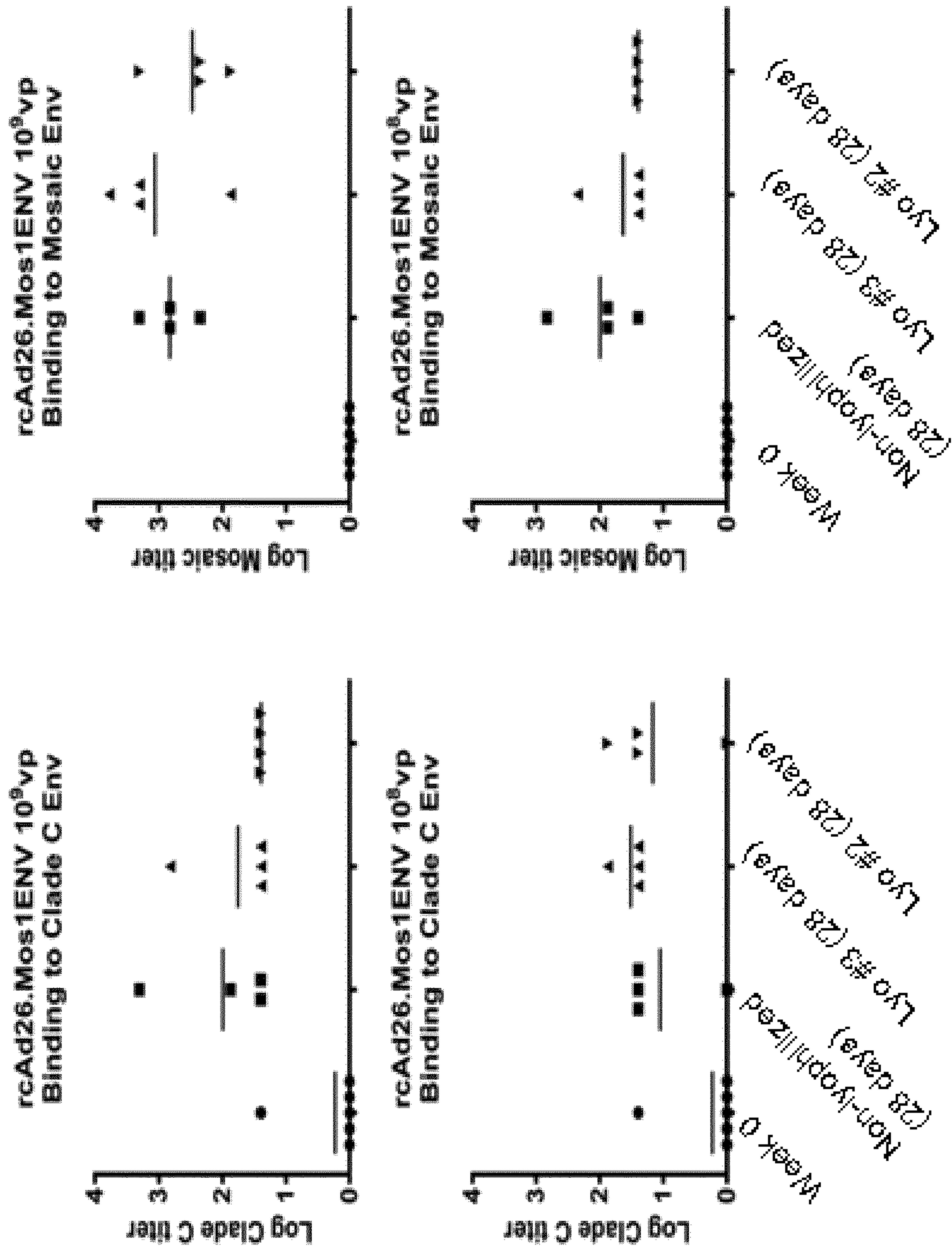


Fig. 11 B

Ad26.Mos1ENV IM					Ad26.Mos1ENV IN					Ad26.Mos1ENV IM					Ad26.Mos1ENV IN								
Oral swabs					Rectal swabs					Serum					Serum								
Mouse	d0	d7	d14	d21	d28	Mouse	d0	d7	d14	d21	d28	Mouse	d0	d7	d14	d21	d28	Mouse	d0	d7	d14	d21	d28
1-1	0	0	0	0	0	1-1	0	0	0	0	0	1-1	0	0	0	0	0	1-1	0	0	0	0	0
1-2	0	0	0	0	0	1-2	0	0	0	0	0	1-2	**	0	0	0	0	1-2	**	0	0	0	0
1-3	0	0	0	0	0	1-3	0	0	0	0	0	1-3	**	0	0	0	0	1-3	**	0	0	0	0
1-4	0	0	0	0	0	1-4	0	0	0	0	0	1-4	**	0	0	0	0	1-4	**	0	0	0	0
Ad26.Mos1ENV IN					Ad26.Mos1ENV IN					Ad26.Mos1ENV IN					Ad26.Mos1ENV IN								
Oral swabs					Rectal swabs					Serum					Serum								
Mouse	d0	d7	d14	d21	d28	Mouse	d0	d7	d14	d21	d28	Mouse	d0	d7	d14	d21	d28	Mouse	d0	d7	d14	d21	d28
2-1	0	0	0	0	0	2-1	0	0	0	0	0	2-1	0	0	0	0	0	2-1	0	0	0	0	0
2-2	0	0	0	0	0	2-2	0	0	0	0	0	2-2	**	0	0	0	0	2-2	**	0	0	0	0
2-3	0	0	0	0	0	2-3	0	0	0	0	0	2-3	**	0	0	0	0	2-3	**	0	0	0	0
2-4	0	0	0	0	0	2-4	0	0	0	0	0	2-4	**	0	0	0	0	2-4	**	0	0	0	0
rcAd26.Mos1ENV IM					rcAd26.Mos1ENV IM					rcAd26.Mos1ENV IM					rcAd26.Mos1ENV IM								
Oral swabs					Rectal swabs					Serum					Serum								
Mouse	d0	d7	d14	d21	d28	Mouse	d0	d7	d14	d21	d28	Mouse	d0	d7	d14	d21	d28	Mouse	d0	d7	d14	d21	d28
3-1	0	0	0	0	0	3-1	0	0	0	0	0	3-1	0	0	0	0	0	3-1	0	0	0	0	0
3-2	0	0	0	0	0	3-2	0	0	0	0	0	3-2	**	0	0	0	0	3-2	**	0	0	0	0
3-3	0	0	0	0	0	3-3	0	0	0	0	0	3-3	**	0	0	0	0	3-3	**	0	0	0	0
3-4	0	0	0	0	0	3-4	0	0	0	0	0	3-4	**	0	0	0	0	3-4	**	0	0	0	0
rcAd26.Mos1ENV IN					rcAd26.Mos1ENV IN					rcAd26.Mos1ENV IN					rcAd26.Mos1ENV IN								
Oral swabs					Rectal swabs					Serum					Serum								
Mouse	d0	d7	d14	d21	d28	Mouse	d0	d7	d14	d21	d28	Mouse	d0	d7	d14	d21	d28	Mouse	d0	d7	d14	d21	d28
4-1	0	0	0	0	0	4-1	0	0	0	0	0	4-1	0	0	0	0	0	4-1	0	0	0	0	0
4-2	0	0	0	0	0	4-2	0	0	0	0	0	4-2	**	0	0	0	0	4-2	**	0	0	0	0
4-3	0	0	0	0	0	4-3	0	0	0	0	0	4-3	**	0	0	0	0	4-3	**	0	0	0	0
4-4	0	0	0	0	0	4-4	0	0	0	0	0	4-4	**	0	0	0	0	4-4	**	0	0	0	0
Control	6.1E+06	4.0E+06	3.6E+06	2.9E+06	2.0E+06	Control	5.0E+06	5.2E+06	1.6E+06	2.8E+06	1.3E+06	Control	4.2E+06	5.2E+06	5.2E+06	5.2E+06	4.8E+06	Control	4.2E+06	5.2E+06	5.2E+06	5.2E+06	4.8E+06

Fig. 12

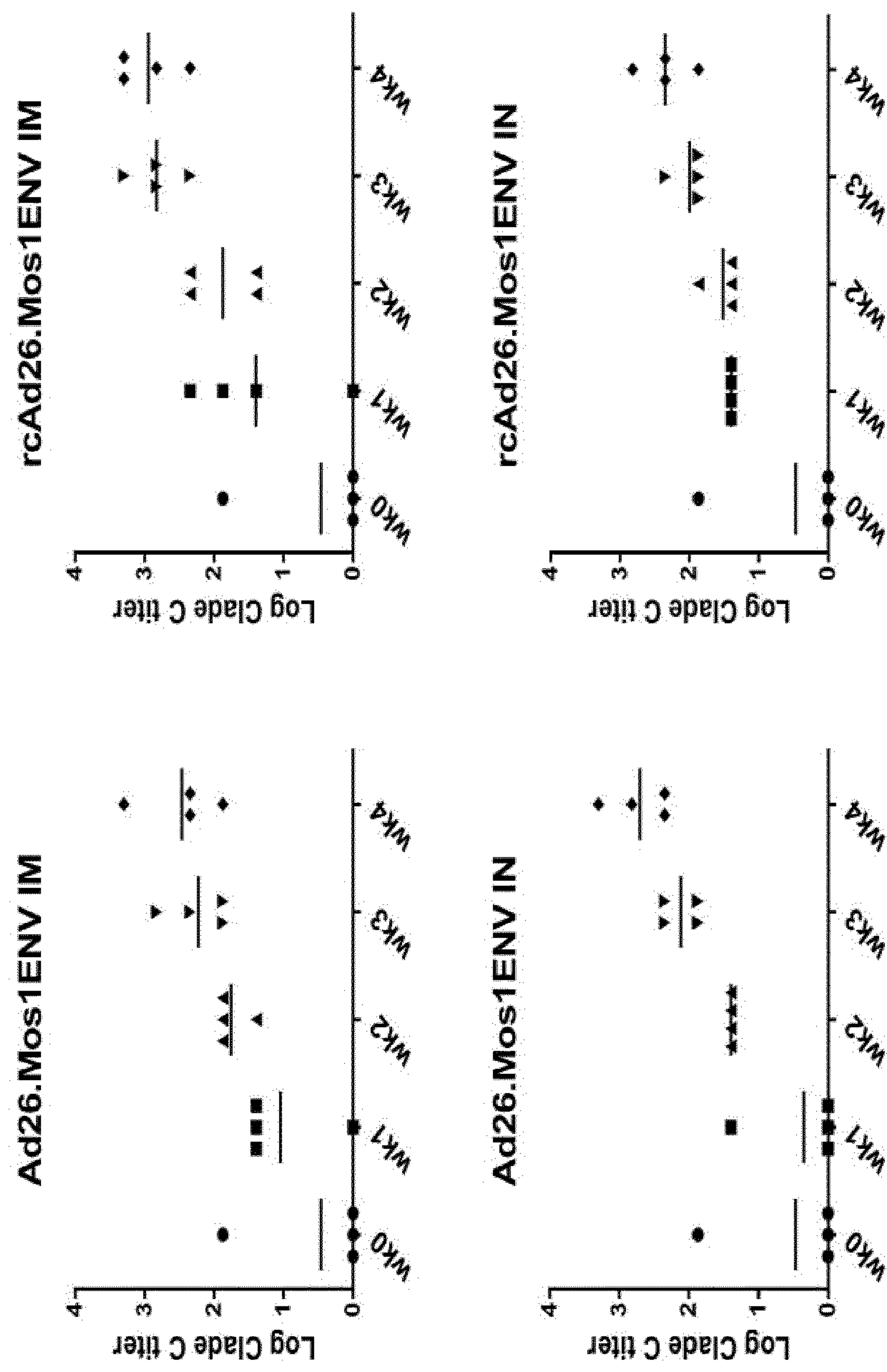


Fig. 13 A

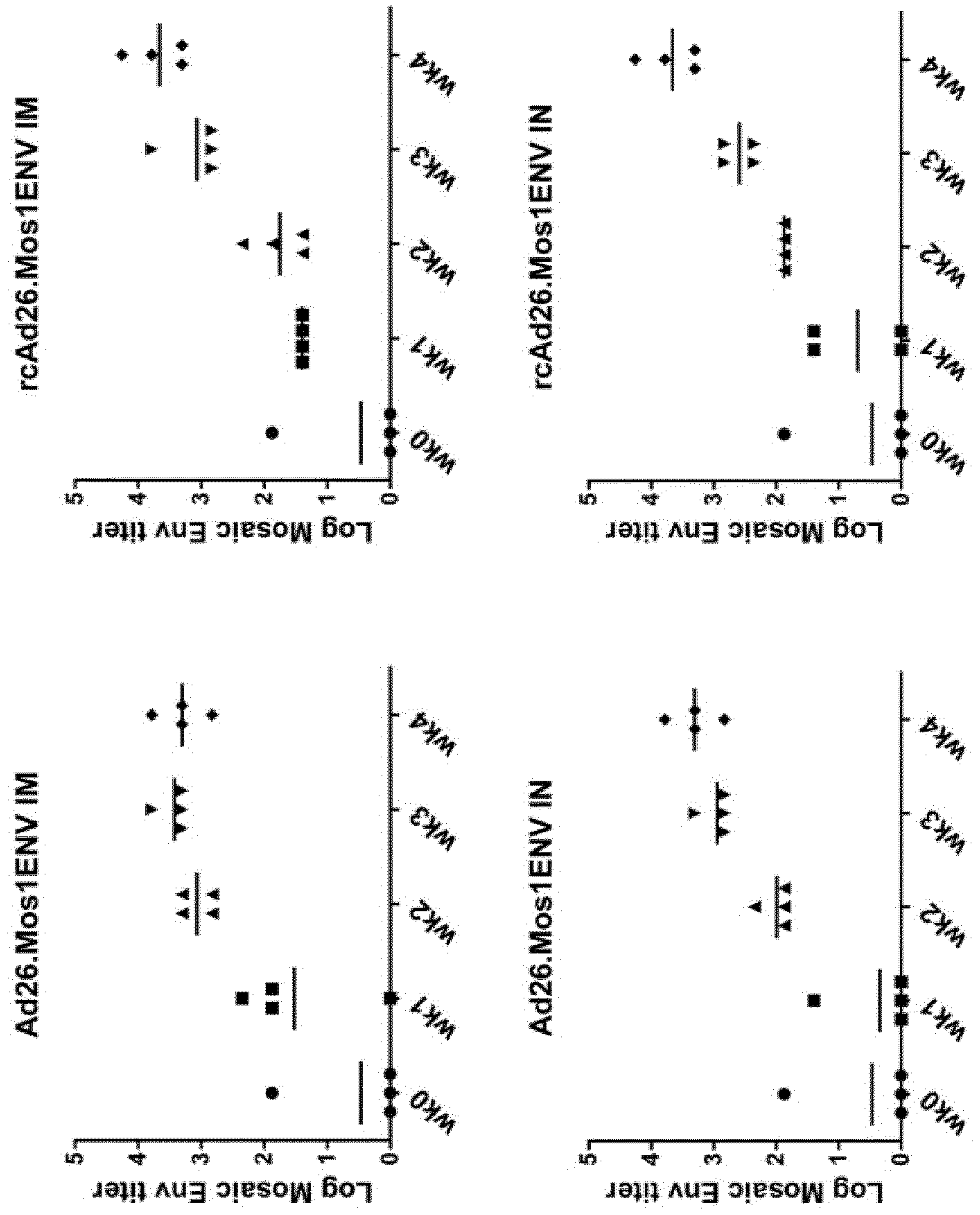


Fig. 13 B

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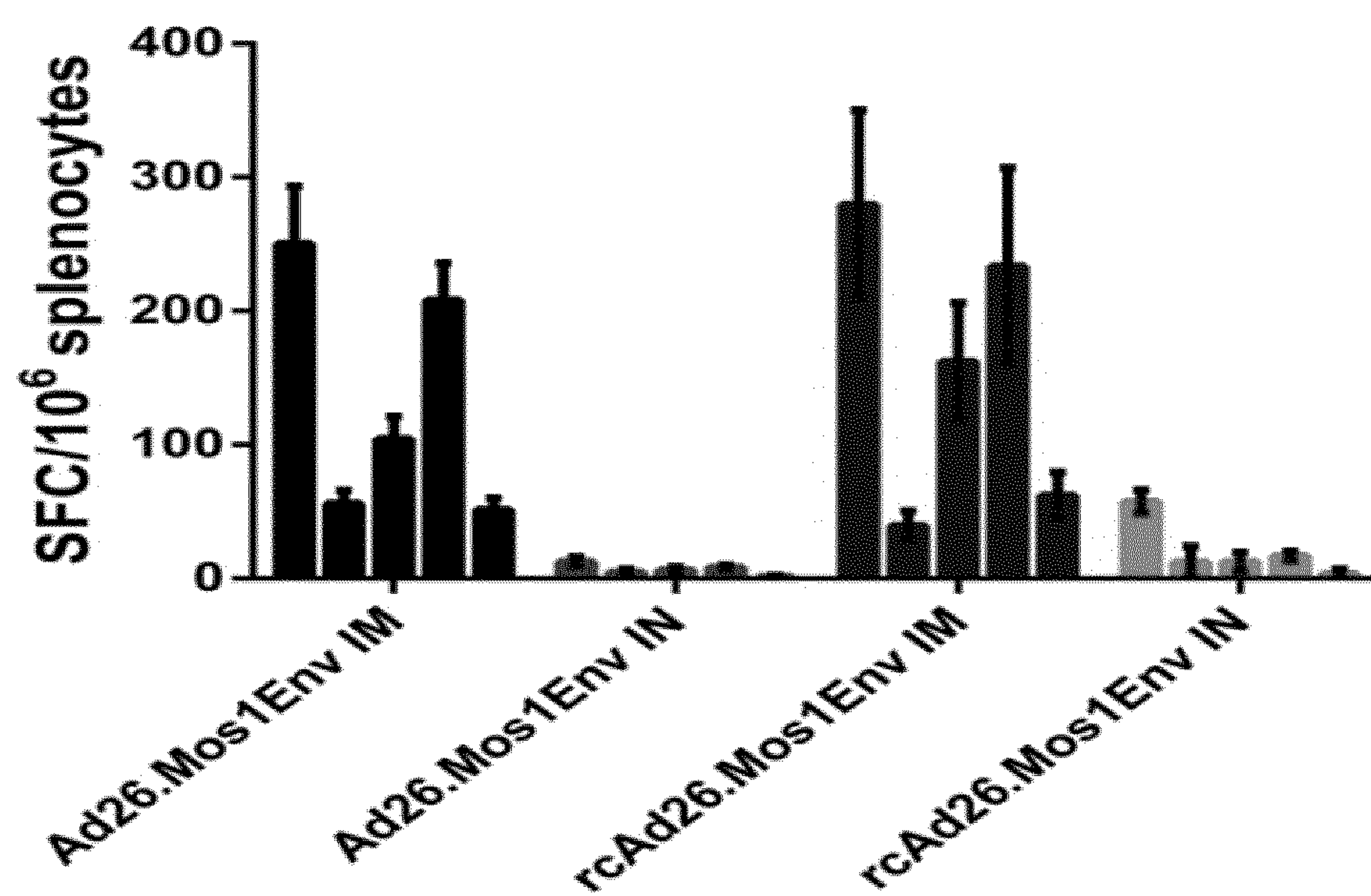


Fig. 14

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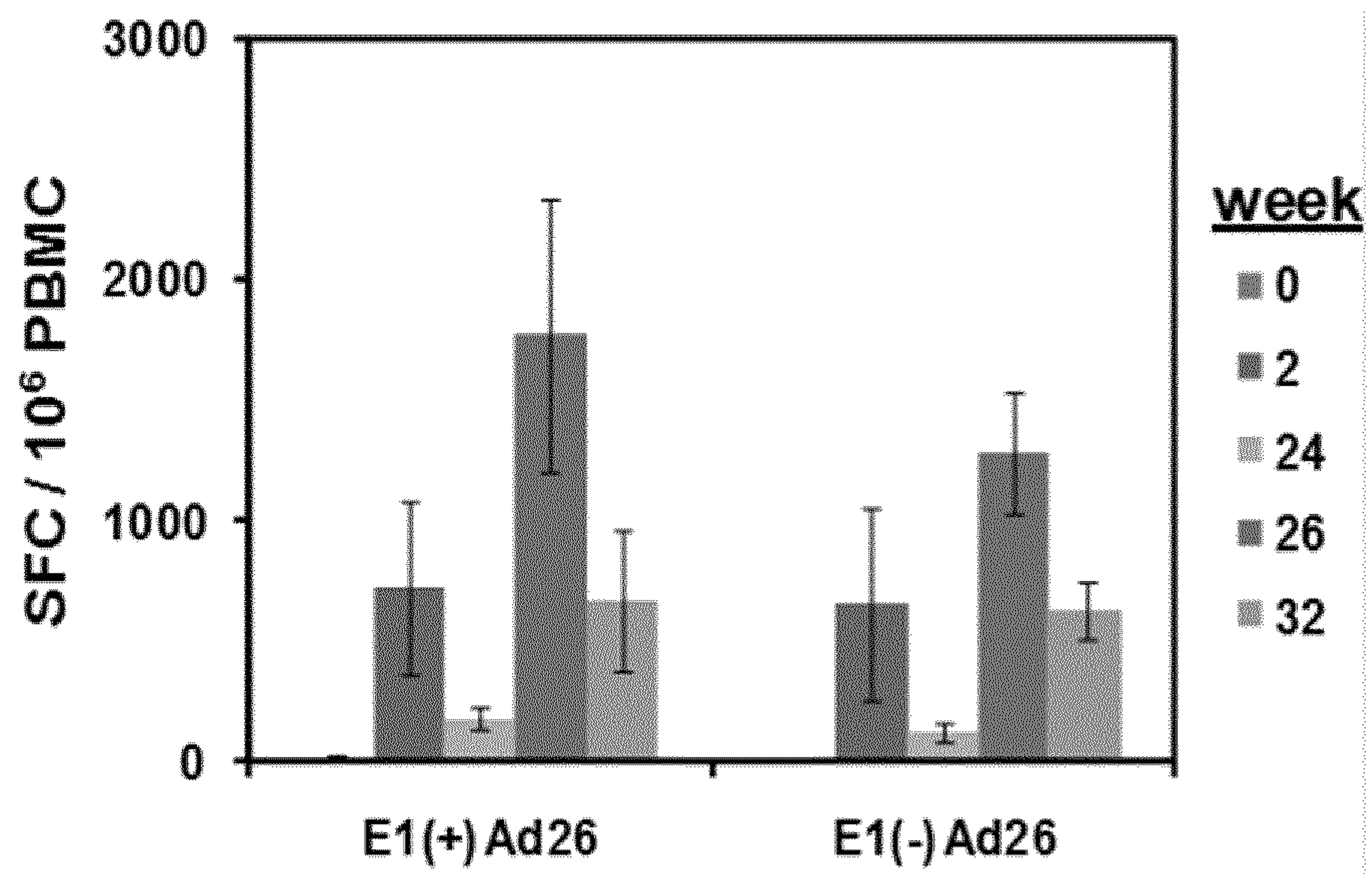


Fig. 15 A

RT-PCR Results on Serum Samples from Monkeys Immunized with Replicating E1(+) and Non-Replicating E1(-) Ad26-SIVGag Vectors.

Serum Monkey	Wk-1	Wk1	Wk4	Wk6	Wk8	Wk10	Wk12	Wk16
415-08	0	0	0	0	0	0	0	0
421-08	0	0	0	0	0	0	0	0
451-08	0	0	0	0	0	0	0	0
427-08	0	0	0	0	0	0	0	0
429-08	0	0	0	0	0	0	0	0
432-08	0	0	0	0	0	0	0	0
Control	3.0E+05	2.8E+05	4.4E+05	3.2E+05	3.8E+05	3.6E+05	3.7E+05	3.1E+05

Fig. 15 B

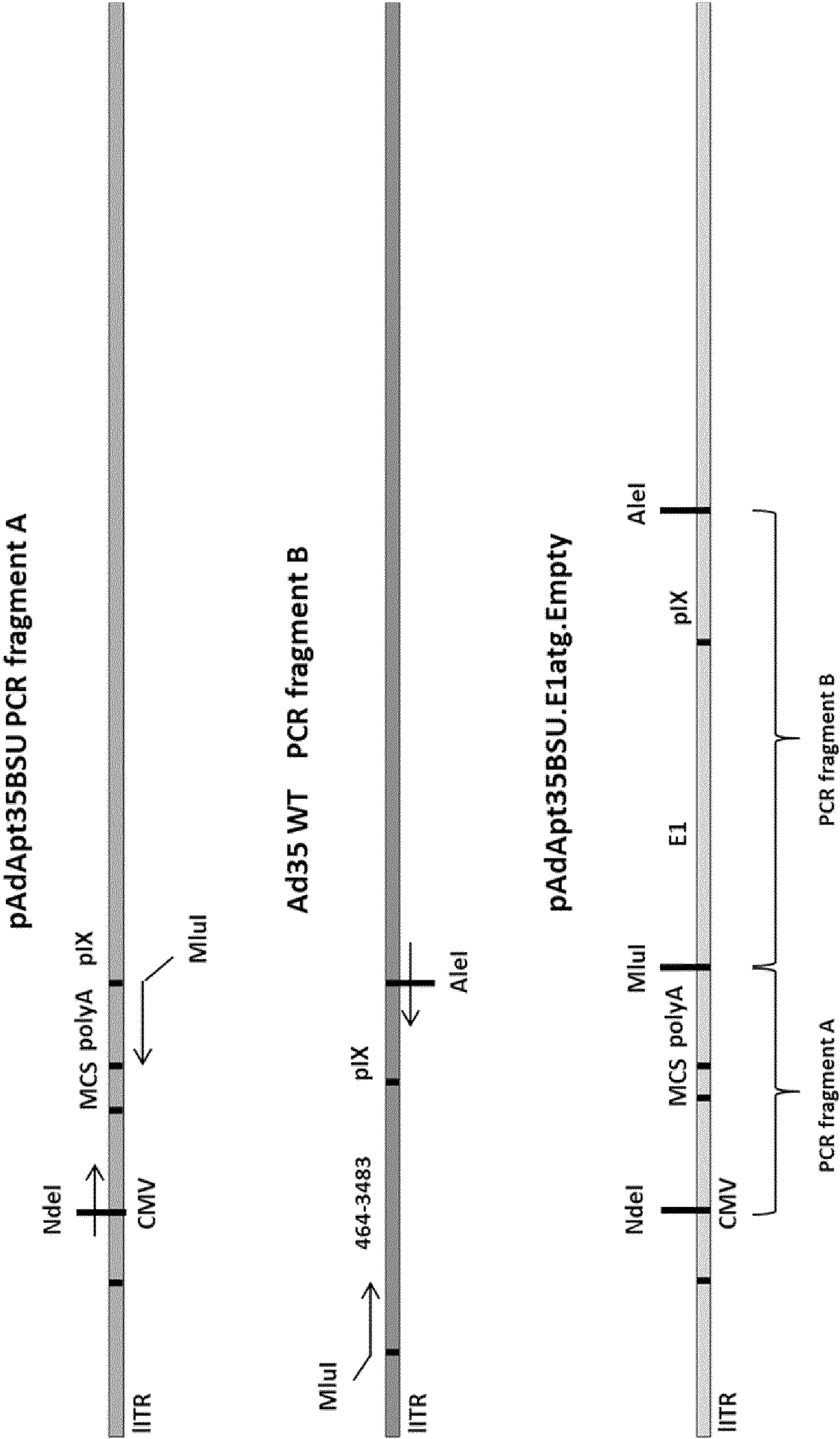
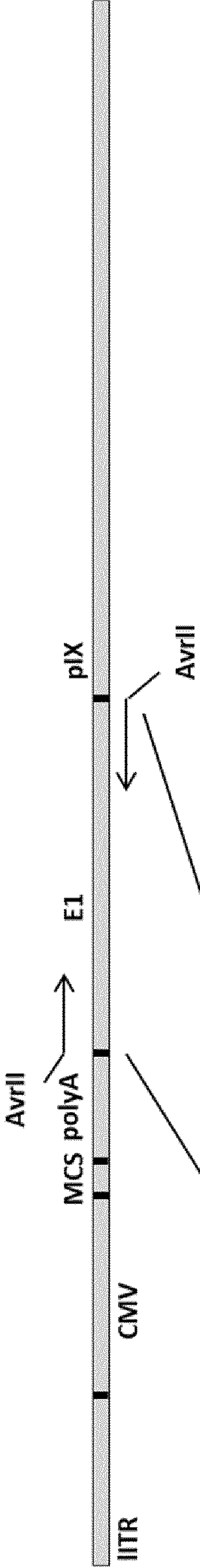
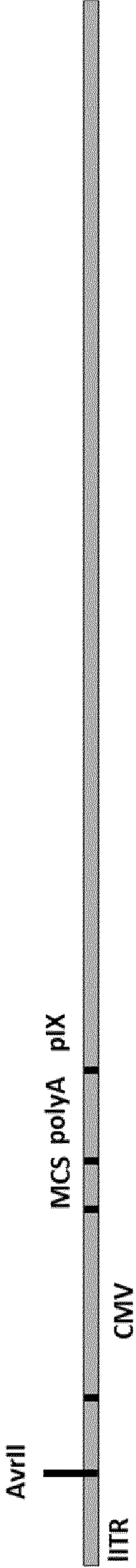


Fig. 16 A

pAdApt35BSU.E1atg.Empty



pAdApt35BSU



pAdApt35BSU.E1btg.Empty



Fig. 16 B

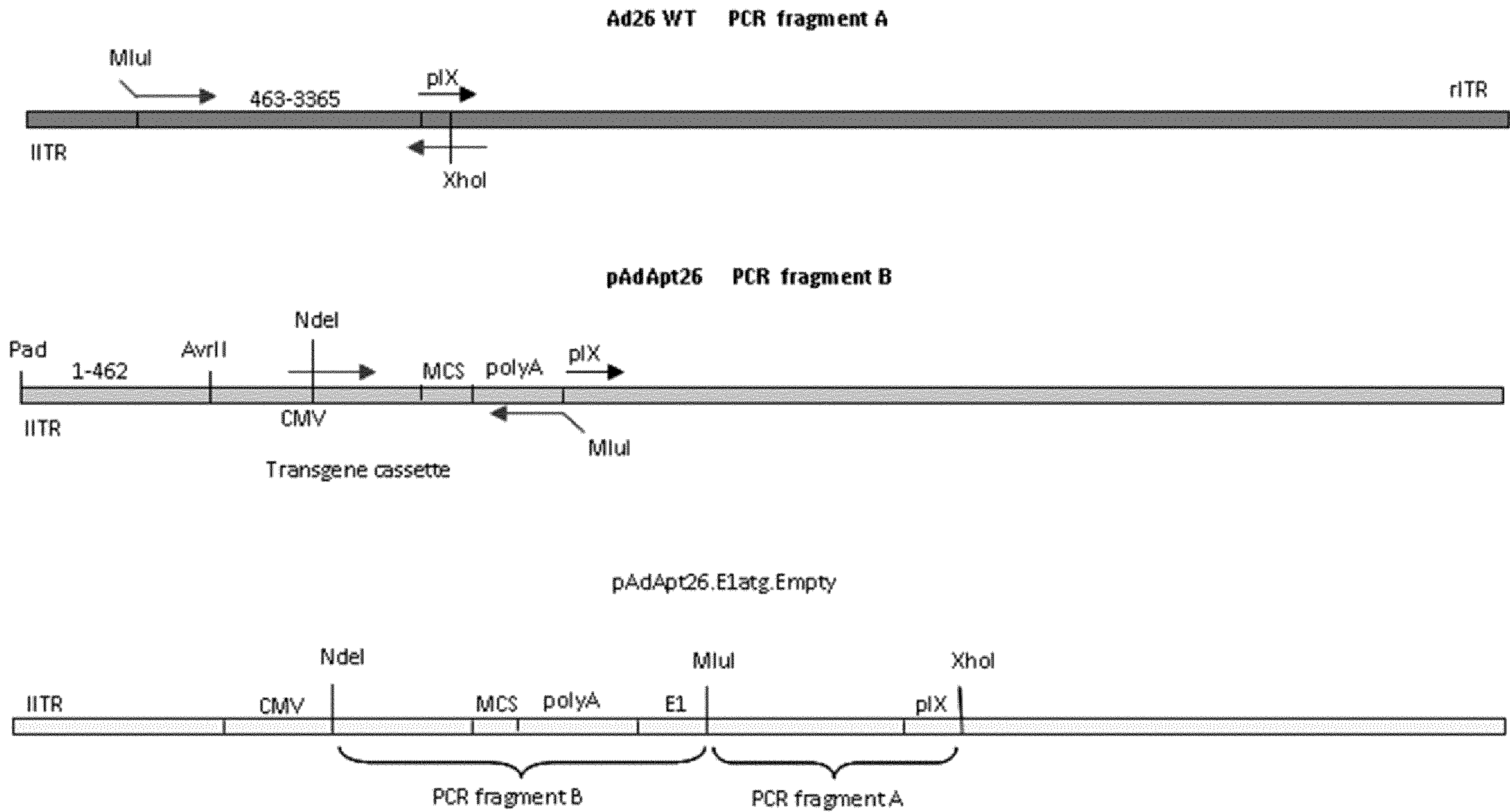


Fig. 2