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(54) Title: IMPROVED REVERSE GENETICS FOR SINGLE STRAND NEGATIVE RNA VIRUSES

(57) Abstract: The invention relates to an *in vitro* method of rescuing negative RNA virus from low virulent virus strain(s) or lentogenic-like virus strain(s) belonging to the order Mononegavirales including *Bomaviridae*, *Filoviridae*, *Myonnaviridae*, *Nyamiviridae*, *Paramyxoviridae*, *Pneumoviridae*, and *Rhabdoviridae* families, preferably belonging to the *Paramyxoviridae* family, comprising at least the steps of: (i) co-transfecting host cells with a two-plasmid system comprising a. a pGenome plasmid comprising at least a sequence encoding a virus genome from low virulent virus strain(s) or lentogenic-like virus strain(s) and b. a pNPL helper plasmid comprising at least the sequences encoding the structural viral proteins nucleocapsid protein (N), phosphoprotein (P) and large protein (L), (ii) culturing host cells under conditions for replication and transcription of the virus, and (iii) recovering the rescued negative strand RNA viruses.



Improved reverse genetics for single strand negative RNA viruses

FIELD OF THE INVENTION

The present invention relates to an improved reverse genetics system for rescuing negative
5 RNA virus from low virulent virus strains, in particular for an avian paramyxovirus from
Avulavirus genus, the Newcastle Disease Virus (NDV) and another paramyxovirus, Peste des
Petits Ruminants Virus (PPRV) from Morbillivirus genus.

BACKGROUND OF THE INVENTION

10 Reverse genetics have been widely used to edit virus genomes and rescue modified viruses
with altered functions or new vaccine properties (1). The general scheme for this method
relies on the cloning of the complete viral genome, segmented or not, in one or several
plasmids under promoters that will generate negative RNAs. Complementary plasmids are
produced to express viral proteins that will take over the transcribed viral genome (1). Since
15 the transfection of a high number of plasmids of different size into one cell is tricky but
indispensable for successful virus rescue, several groups have tried to improve the system
either by reducing the number of plasmids to be used (2, 3) or by generating cells that
constitutively express the viral polymerase complex. However, the latter is a more
complicated and time-consuming process.

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Within Paramyxovirus, conventional reverse genetics require the transfection of a minimum of
four plasmids: three to reconstruct the viral polymerase complex that replicates and
expresses the virus genome delivered by a fourth plasmid. The successful transfection of four
or more plasmids of different sizes into one cell and the subsequent generation of at least
25 one viable and replicable viral particle is a rare event, which explains the low rescue
efficiency, especially of low virulent viruses with inherently lower replication capacity. Yet the
generation of low virulent viruses by reverse genetics is of particular interest for the
management of animal health since it can provide improved attenuated vaccine strains.

30 The Newcastle Disease Virus (NDV), which is an avian *Paramyxoviridae* from *Avulavirus*
genus, becomes again an animal health priority, at least in the countries where it is highly
prevalent (Africa and Asia), because of the progressive reduction of the vaccination efficacy
in the field currently ascribed to an antigenic drift of the virus. This issue mobilizes

researchers in the world to generate improved vaccines (e.g. antigenically closer to circulating viruses) to prevent or treat its propagation. Reverse genetics has long been used in research on NDV virulence, vaccine generation, oncolytic applications, virion assembly, etc. (13-16). The conventional reverse genetics of NDV involves four different plasmids (13).

5 The complete genome and the N, P and L genes are cloned into plasmids downstream from polymerase promoters CMV or T7. These plasmids are co-transfected into eukaryotic cells and transcribed directly by the cells under the control of CMV promoter or indirect transfection, by the bacteriophage T7 RNA polymerase (17, 18). Since NDV reverse genetics was first developed in the 1990s, the technology has been extensively used in research (13).

10 For example, virulent factors of the virus have been identified and some gene functions have been clarified (10, 13, 20, 21). Different modified vaccine candidates have also been generated (14, 22). Other pathogen genes have been inserted into the full genome of NDV and recombinant strains rescued using this technique as vaccine candidates for other diseases (23, 24). Last but not least, the genome of the NDV virus has been engineered by

15 reverse genetics to enhance the oncolytic ability of the virus (16). In this context, virus rescue has been a quite important issue. Different modifications were proposed to improve NDV reverse genetics from its very beginning (1). For instance, in reverse genetics based on the T7 polymerase promoter for RNA transcription and protein expression, T7 RNA polymerase (T7pol) transgenic cell lines were generated to replace the use of a recombinant T7pol-

20 fowlpox virus, thus eliminating the risk of contamination of the rescued virus by the fowlpox virus (18). Other authors used an additional plasmid to express T7pol (25). In other systems, the T7pol promoter was replaced by the CMV promoter, which renders the reverse genetics independent of the T7pol, simplifies the molecular constructions and extends the types of cell lines that can be used to rescue the viruses (17).

25

In another part, Peste des petits ruminants (PPR) is a highly contagious viral disease of sheep, goats and wild small ruminants that causes mortality rates that may be as high as 90% in naïve populations. The disease is caused by a morbillivirus, *Peste des petits ruminants virus* (PPRV), which is an enveloped ribonucleic acid (RNA) virus with a

30 monosegmented genome of negative sense, belonging to the genus *Morbillivirus* in the family *Paramyxoviridae*. Other members of this genus include measles virus (MV), a serious human pathogen, canine distemper virus (CDV) affecting animals of the family Canidae, phocine distemper virus (PDV) and cetacean morbilliviruses (CMV) which affect marine mammals.

This disease has an important economic impact in countries affected. PPR is currently present in Africa, Middle East, and Asia. With multiple outbreaks recorded in Turkey, Georgia and the Maghreb region, it is now at the door of Europe. PPR has been flagged as the next target for global eradication through massive vaccination campaigns coordinated by the world organization for animal health (OIE). This effort may last for more than 15 years and cost billions of dollars.

Presently, the most widely used vaccine is the PPRV Nigeria 75/1 vaccine strain developed by the CIRAD (Centre de coopération internationale en recherche agronomique pour le développement) in 1989 in collaboration with the Institute for Animal Health at Pirbright UK. It was produced by attenuating the virulent strain PPRV Nigeria 75/1 through multiple passages in cell cultures. In particular, this virus was isolated originally from a sick goat on primary lamb kidney cell culture in Nigeria and attenuated by serial passages on Vero cells (Diallo et al., 1989). This vaccine is perfectly safe and provides a good protection to animals during their economic life (i.e. at least 3 years). The massive vaccination campaigns planned by the OIE depend on these attenuated vaccines. However, these vaccines do not permit to differentiate by serology between vaccinated and infected animals (Differentiating Infected from Vaccinated Animals, also referring to DIVA vaccine). Therefore, a robust and efficient reverse genetic method is necessary to quickly develop new DIVA vaccines.

However, until now, reverse genetics systems were all based on the use of four different plasmids to deliver the minimum elements of the replicative form of the virus: the complete genome and the N, P, and L proteins of the virus. Although generally successful, this 4-plasmids system has to get round the difficulty of sending four different size plasmids into the same cell to be able to generate an infectious clone. This difficulty is increased by the fact that the plasmid containing the complete genome is large and its transfection efficiency can be affected. In addition, the success rate of generating an infectious clone from four plasmids transfected, inherently low in this system is even more reduced when the virus has a low replication capacity *in vitro*.

So there is still a need of providing a reverse genetics system for rescuing negative RNA virus from low virulent virus strains *in vitro*, in particular for virus belonging to the *Paramyxoviridae* family, in particular from *Avulavirus* genus such as NDV and *Morbillivirus* genus such as PPRV.

The inventions disclosed and provided herein meet these and other needs. In particular, the present invention provides an improved reverse genetics system developed for

single strand negative RNA viruses, in particular for an avian paramyxovirus, the Newcastle Disease Virus (NDV) and for another paramyxovirus, Peste des Petits Ruminants Virus (PPRV), in which the number of plasmids was reduced from four to two. Compared to the conventional method, the 2-plasmids system enables earlier and increased production of rescued viruses and, in addition, makes it possible to rescue viruses that it was not possible to rescue using the 4-plasmids system.

The 2-plasmids system developed for NDV reverse genetics not only simplifies the transfection procedure, reduces the number of plasmids to be transfected and requires less time to achieve successful rescue, but also increases the efficiency for lentogenic-type viruses compared to the conventional 4-plasmids system. Using this improvement in reverse genetics for other viruses may be equally successful.

SUMMARY OF THE INVENTION

A first object of the invention is an *in vitro* method of rescuing negative RNA virus from low virulent virus strain(s) or lentogenic-like virus strain(s), comprising at least the steps of:

- (i) co-transfecting host cells with a two-plasmid system comprising
 - a. a pGenome plasmid comprising at least a sequence encoding a virus genome from low virulent virus strain(s) or lentogenic-like virus strain(s) and
 - b. a pNPL helper plasmid comprising at least the sequences encoding the structural viral proteins nucleocapsid protein (N), phosphoprotein (P) and large protein (L),
- (ii) culturing host cells under conditions for replication and transcription of the virus, and
- (iii) recovering the rescued negative strand RNA viruses.

In a particular and preferred embodiment, low virulent virus strain(s) or lentogenic-like virus strain(s) belong to the *Paramyxoviridae* family.

The invention also relates to an eukaryotic host cell transformed (transfected) with the two-plasmid system according to the invention.

Another object of the invention is a rescued negative RNA virus isolated from the eukaryotic cell transformed according to the method as defined above, preferably after 1 day post-transfection, more preferably after 3 days post-transfection.

The invention also provides a helper plasmid pNPL comprising at least the sequences of the structural viral proteins of a virus belonging to the *Paramyxoviridae* family, in particular the Newcastle Disease Virus (NDV) or the Peste des Petits Ruminants Virus (PPRV), consisting of nucleocapsid protein (N), phosphoprotein (P) and large protein (L), under control of a promoter, in particular a CMV promoter.

Another object of the invention is a kit for *in vitro* direct rescue of negative RNA virus from low virulent virus strain(s) or lentogenic-like virus strain(s) comprising at least:

- (i) a pGenome plasmid comprising at least a sequence encoding the virus genome from low virulent virus strain(s) or lentogenic-like virus strain(s), preferably belonging to the *Paramyxoviridae* family, in particular the Newcastle Disease Virus (NDV) or the Peste des Petits Ruminants Virus (PPRV),
- (ii) a pNPL helper plasmid comprising at least the sequences of the structural viral proteins of a negative RNA virus, preferably belonging to the *Paramyxoviridae* family, in particular the Newcastle Disease Virus (NDV) or the Peste des Petits Ruminants Virus (PPRV), consisting of nucleocapsid protein (N), phosphoprotein (P) and large protein (L),
- (iii) optionally host cells,
- (iv) optionally culture medium for transfecting and culturing host cells, and
- (v) optionally well-plates.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

In vitro method of virus rescue

In a first embodiment, the *in vitro* method of rescuing negative RNA virus from low virulent virus strain(s) or lentogenic-like virus strain(s) according to the invention comprises at least the steps of:

- (i) co-transfecting host cells with a two-plasmid system comprising
 - a. a pGenome plasmid comprising at least a sequence encoding the virus genome from low virulent virus strain(s) or lentogenic-like virus strain(s) and
 - b. a pNPL helper plasmid comprising at least the sequences encoding the structural viral proteins nucleocapsid protein (N), phosphoprotein (P) and large protein (L),
- (ii) culturing host cells under conditions for replication and transcription of the virus, and

- (iii) recovering the rescued negative strand RNA viruses.

In an alternative embodiment according to the invention for the *in vitro* method:

- 5 a) either the pGenome plasmid comprises a sequence encoding a virus genome which is partially deleted or mutated, and the host cell is optionally modified for expressing the said deleted gene(s),
- b) either the pNPL helper plasmid is substituted by a helper plasmid comprising one or two sequences selected from the group consisting of sequences encoding N, P and L proteins, and the host cell is modified to complement the expression of missing
- 10 protein(s).

In a particular and preferred embodiment, low virulent virus strain(s) or lentogenic-like virus strain(s) belong to the *Paramyxoviridae* family.

- 15 The co-transfecting technologies for step (i) and culturing conditions for step (ii) are well known from the man skilled in the art.

The term 'rescuing a virus' according to the invention encompasses any process well known from the man skilled in the art allowing the generation of an infectious viral clone from a

20 cDNA of the virus genome.

The term 'negative RNA virus' according to the invention encompasses negative-sense single-stranded RNA viruses that require a RNA replicase, also known as RNA-dependent RNA polymerase (RdRp), to initiate replication of their genome. Such viruses belong to the

25 order Mononegavirales including *Bornaviridae*, *Filoviridae*, *Mymonaviridae*, *Nyamiviridae*, *Paramyxoviridae*, *Pneumoviridae*, *Rhabdoviridae* families.

In a particular embodiment, such viruses all comprise sequences encoding N, P and L proteins and belong to *Bornaviridae*, *Filoviridae*, *Mymonaviridae*, *Nyamiviridae*, *Paramyxoviridae*, *Pneumoviridae*, or *Rhabdoviridae* families.

- 30 In a particular and preferred embodiment, negative RNA virus is a paramyxovirus, preferably an avian paramyxovirus, and more preferably the Newcastle Disease Virus (NDV).

In another particular and preferred embodiment, negative RNA virus is a paramyxovirus from *Morbillivirus* genus, and more preferably Peste des Petits Ruminants Virus (PPRV).

5 The term 'low virulent virus strains or lentogenic-like virus strains' according to the invention encompasses live or recombinant attenuated viruses in the target species.

Such 'low virulent virus strains or lentogenic-like virus strains' have a reduced pathogenic phenotype compared to the virulent virus strains of the same genus. A reduced pathogenic phenotype encompasses a reduced infection capacity and/or a reduced replication capacity, and/or a reduced and/or restricted tissue tropism, and/or a default or defect in the assembly
10 of the viral particles, more particularly a reduced infection capacity.

In a particular embodiment for *Morbillivirus* genus, a low virulent virus strain is the PPRV Nigeria 75/1 vaccine strain disclosed in Diallo et al., 1989 (Genbank accession number KY628761.1 or X74443.2, 15948 bp linear RNA, SEQ ID N0: 31).

So, in a particular embodiment, the invention relates to an *in vitro* method of rescuing
15 negative RNA virus wherein the low virulent virus strain is selected from PPRV Nigeria 75/1 strain (SEQ ID N0:31) or PPRV recombinant Nigeria 75/1 strain.

In another particular embodiment for *Avulavirus* genus, a low virulent virus strain is the NDV LaSota strain (Genbank accession numbers AY845400.2, AF077761 or JF950510, 15186 bp linear RNA, SEQ ID N0: 12).

20 For avian paramyxoviruses, and in particular for NDV, the molecular basis for the different level of pathogenicity (more than 10 different genotypes) is known to be linked to the sequence of the cleavage site of the precursor of the fusion protein F. At this position, a pathogenic NDV strain for example has at least one extra pair of basic amino-acids motif ¹¹²X-R-X-R/K-R-F¹¹⁷ and can be cleaved by a wide range of proteases of the furin family in
25 different host cells.

For morbilliviruses, virulence is not determined by a single genetic element. In contrast, several mutations accumulated in the leader, trailer and viral proteins have been shown to support virulence for Measles, rinderpest and canine distemper viruses. Genetic determinisms of PPRV virulence are even less characterized. According to the invention, the
30 low virulent or lentogenic virus strains (empirically attenuated or vaccine strain, or genetically engineered vaccine strain recombinant) for avian paramyxovirus have a genotype coding for

a F protein cleavage site which has less than 4 basic amino acids, in particular less than 3 basic amino acids, and preferably only two basic amino acids, for example in amino acids positions 112-117 for NDV virus. It is also said that the F protein cleavage site is modified or mutated (Fmu).

- 5 In some embodiments, the low virulent virus strain is selected from the group consisting of virus strains having a genotype with a lentogenic-like F protein cleavage site, in particular NDV LaSota strain (SEQ ID N0:12), or NDV recombinant strains such as LaSota recombinant strain (LaSota/M-Fmu SEQ ID N0: 14), or attenuated recombinant MG-725 strain (MG-725/Fmu SEQ ID N0: 9).
- 10 So, in a particular embodiment, the invention relates to an *in vitro* method of rescuing negative RNA virus, wherein the low virulent virus strain is selected from NDV LaSota strain (SEQ ID N0:12) or NDV recombinant LaSota strain.

The term 'genotype with a lentogenic-like F protein cleavage site' according to the invention
15 encompasses genotype wherein the amino-acid positions 112-117 of the precursor of the F protein contain less than 4 basic amino acids, in particular less than 3 basic amino acids, and preferably only two basic amino acids.

For the NDV, the velogenic strains have five basic amino acids, while the lentogenic strains have two basic amino acids (Fig. 3). This difference makes the F protein of virulent strains
20 more prone to be cleaved by various proteases present in various tissues and the virus is then activated to amplify whereas the F protein of attenuated strains is only cleaved in environments like the digestive and respiratory tracts or *in vitro*, in cell culture medium containing trypsin.

25 *pGenome*

- in a particular embodiment, the pGenome plasmid comprises at least a sequence encoding the virus genome from low virulent virus strain(s) or lentogenic-like virus strain(s), in particular at least a sequence encoding the genome Newcastle Disease Virus (NDV) from low virulent strain(s) or lentogenic-like virus strain(s), such as LaSota strain.
- 30 In a preferred embodiment, the sequence encoding the virus genome from low virulent virus strains or lentogenic-like virus strains according to the invention comprises a modified F protein cleavage site, in particular a sequence of formula (I) $^{112}X_1-X_2-X_3-X_4-X_5-X_6^{117}$ wherein

X₂ and X₅ are independently arginine (R) or lysine (K), preferably arginine (R) and X₁, X₃, X₄, X₆ are independently selected from the group consisting of non-basic amino acids.

In a preferred embodiment, the sequence encoding the virus genome from low virulent virus strains or lentogenic-like virus strains according to the invention comprises a modified F
5 protein cleavage site, in particular a sequence GRQGRL (SEQ ID N0: 18).

In a particular embodiment, the pGenome plasmid comprises at least a sequence encoding the virus genome of Newcastle Disease Virus (NDV) from a low virulent virus strain, such as LaSota strain (Genbank accession numbers Genbank accession numbers AY845400.2,
10 AF077761 or JF950510, 15186 bp linear RNA, SEQ ID N0:12).

Examples of genes and plasmids sequences illustrated in the present invention are disclosed further in the description in the table 1.

The NDV belongs to the *Avulavirus* genus in the *Paramyxoviridae* family (4, 5). The genome is composed of a 15 kb negative-sense single-stranded RNA molecule with six coding
15 segments surrounded by the leader and trailer viral polymerase promoters (6-8). This genome structure (3'-Leader-N-P-M-F-HN-L-Trailer-5') encodes six structural viral proteins: nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), large protein (L), and two nonstructural proteins – V and W proteins, respectively (9, 10). Among these proteins, N, P and L form the viral polymerase
20 complex replicates and transcribes the viral genome (10). These three proteins play a crucial role in virus rescue by reverse genetics (11-13).

In another embodiment, the pGenome plasmid comprises at least a sequence encoding the virus genome from low virulent virus strain(s) of *Morbillivirus* genus, preferably Peste des
25 Petits Ruminants Virus (PPRV), such as PPRV Nigeria 75/1 strain as illustrated in the examples (SEQ ID N0:31).

In an alternative embodiment, the pGenome plasmid comprises at least a sequence encoding a virus genome which is partially deleted and/or mutated and the host cell is optionally
30 modified to complement the said deleted gene(s).

A "mutation" as used herein, refers to a change in nucleic acid relative to a reference sequence (which is preferably a naturally-occurring normal or « wild-type » or « reference » sequence), and includes translocations, deletions, insertions, and substitutions mutations. A

mutation by “substitution” as used with respect to amino acids, refers to the replacement of one amino acid residue by any other amino acid residue, excepted the substituted amino acid residue. Advantageously, small amino acid residues are used for substitution in order to limit any effect on the overall protein structure.

- 5 In particular, the sequence encoding a virus genome within the pGenome comprises a mutation within the cleavage site of the F protein, to be lentogenic-like as the LaSota strain.

In a particular embodiment, the pGenome plasmid comprises at least a sequence encoding the virus genome of Newcastle Disease Virus (NDV) from recombinant LaSota strain (LaSota/M-Fmu SEQ ID N0:14).

- 10 In another particular embodiment, the pGenome plasmid comprises at least a sequence encoding the virus genome of Newcastle Disease Virus (NDV) from recombinant MG-725 strain having a lentogenic-like genotype (MG-725/Fmu SEQ ID N0:9).

The man skilled in the art well knows the technologies for preparing plasmid constructions, pGenome and pNPL helper plasmids, as represented in Figure 1 and illustrated in the further
15 examples.

- In a particular embodiment, the pGenome plasmid comprises at least a partial sequence of virus genome of Newcastle Disease Virus (NDV) from low virulent (lentogenic) strain (ex: LaSota) combined with partial sequences from virulent strain (ex: MG-725) mutated within the
20 F protein cleavage site.

- A « host cell modification », as used herein, refers to any genetic modification of the cell allowing permanent or transient expression of deleted and/or mutated gene(s) to complement the said deleted and/or mutated gene(s) in the rescued virus genome. Modifications include insertional cell genome mutagenesis based on transposons or viruses and cell genome
25 editing by specific nucleases (e.g. TALEN or CRIPR/Cas9) and homologous recombination.

Helper plasmid

- The helper plasmid pNPL used in the method of the invention comprises at least the sequences of the structural viral proteins of a virus belonging to the *Paramyxoviridae* family,
30 in particular the Newcastle Disease Virus (NDV) or Peste des Petits Ruminants Virus (PPRV), consisting of nucleocapsid protein (N), phosphoprotein (P) and large protein (L), under control of a promoter, in particular a pCMV promoter.

In a particular embodiment, the pNPL plasmid comprises three independent expression cassettes under a promoter, in particular pCMV promoter to express N, P, L.

In a particular embodiment, the pNPL helper plasmid comprises at least the sequences encoding the structural viral proteins nucleocapsid protein (N) (SEQ ID N0:1), phosphoprotein (P) (SEQ ID N0:2) and large protein (L) (SEQ ID N0:3) of the Newcastle Disease Virus. Preferably, the pNPL helper plasmid comprises the sequence SEQ ID N0:4.

In another particular embodiment for Peste des Petits Ruminants Virus (PPRV), the pNPL helper plasmid comprises at least the sequences encoding the structural viral proteins nucleocapsid protein (N) (SEQ ID N0:26), phosphoprotein (P) (SEQ ID N0:27) and large protein (L) (SEQ ID N0:28) of PPRV.

Alternatively, the pNPL helper plasmid is substituted by a helper plasmid comprising one or two sequences selected from the group consisting of sequences encoding N, P and L proteins, and the host cell is modified to complement the expression of missing protein(s). As an example, the helper plasmid comprises the sequences encoding for N and P proteins and the host cell is modified to complement the expression of L protein.

In some embodiments, the weight ratio between the first plasmid pGenome and the helper plasmid pNPL ranges from 9:1 to 1:9, and is preferably 1:1.

In a particular embodiment, the negative RNA virus is a virus belonging to the *Paramyxoviridae* family, in particular the Newcastle Disease Virus (NDV) or Peste des Petits Ruminants Virus (PPRV).

In some embodiments, the host cells are eukaryotic cells, in particular mammal cells, preferably baby hamster kidney cells (BHK-21) for NDV.

In particular embodiments, the host cells are transfected with an amount of two-plasmid system ranging from 1 µg to 20 µg, in particular from 2 to 20 µg, and preferably from 3 to 5 µg (total amount of both plasmids).

In some embodiments, the *in vitro* method of rescuing negative RNA viruses in host cells additionally comprises a step of virus amplification into chicken embryos. In particular, this additional step is managed between culturing step (ii) and recovering step (iii) of the method. In particular for NDV, the host cells in which the infectious virus clone is generated and their supernatants are collected and injected into 10 day old SPF chicken embryos for virus amplification.

So in a particular embodiment, the *in vitro* method of rescuing negative RNA virus according to the invention additionally comprises a step of amplification of RNA virus into chicken embryos between steps (ii) and step (iii). And in particular, the host cells transformed (transfected) with the two-plasmid system and their supernatants are collected and injected into 10 day old SPF chicken embryos for virus amplification.

The co-transfecting technologies for step (i) and culturing conditions for step (ii) are well known from the man skilled in the art.

In a non-limitative embodiment, the method of rescuing NDV virus may comprise the following steps:

- 1) preparation of the plasmids constructions:
 - . extraction RNA from NDV strain ;
 - . cDNA generation based on viral RNA;
 - . pNPL plasmid construction: amplification of N, P, L genes of NDV from cDNA by PCR and cloning into a plasmid, between a CMV promoter and polyA sequences; then N, P and L genes with CMV promoter and polyA are amplified from pN, pP, and pL by PCR and then cloned into pCMV plasmid to generate pNPL plasmid;
 - . pGenome construction: CMV promoter and polyA replace T7 promoter and terminator of a pKS plasmid and two ribozymes were inserted between CMV promoter and polyA to be the pCMV plasmid; then with PCR and restriction, the full genome of virus is assembled on pCMV plasmid, between both ribozymes, to get the pCMV-NDV (pGenome);
- 2) co-transfection of the host cell with the said plasmids pGenome and pNPL and culture under conditions for replication and transcription of the virus:

BHK-21 cells are seeded on the 6-well plate and cultured at 37°C, 5% CO₂ for overnight; then 1.5µg pCMV-NDV (pGenome) and 1.5µg pNPL (pNPL plasmid) are transfected by Lipofectamin into BHK-21 cells;

3) optionally amplification of the rescued RNA virus:

3 days after transfection, the transfected cells with 200 μ L supernatants are collected and injected into allantoic cavity of 10-days old chicken embryo. This chicken embryo is incubated at 37°C for 3 days and then put at 4°C for overnight;

- 5 4) recovering the rescued virus: the allantoic fluids are harvested. Then, rescuing virus is confirmed with hemagglutination assay (HA) and qRT-PCR.

In a non-limitative embodiment, the method of rescuing PPR virus may comprise the following steps:

- 10 1) preparation of the plasmids constructions:

. extraction RNA from PPRV strain ;

. cDNA generation based on viral RNA;

. pNPL plasmid construction: amplification of N, P, L genes of PPRV from cDNA by PCR and cloning into a plasmid, between a CMV promoter and polyA sequences; then N, P and L

- 15 genes with CMV promoter and polyA are amplified from pN, pP, and pL by PCR and then cloned into pCMV plasmid to generate pNPL plasmid;

. pGenome construction: CMV promoter and polyA replace T7 promoter and terminator of a pKS plasmid and two ribozymes were inserted between CMV promoter and polyA to be the pCMV plasmid; then with PCR and restriction, the full genome of virus is assembled on

- 20 pCMV plasmid, between both ribozymes, to get the pCMV-PPRV (pGenome);

2) co-transfection of the host cell with the said plasmids pGenome and pNPL and culture under conditions for replication and transcription of the virus;

3) optionally amplification of the rescued RNA virus:

4) recovering the rescued virus.

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Eukaryotic host cell

The present invention also provides a eukaryotic host cell transformed (also named transfected) with the two-plasmid system according to the method as defined above.

- In a particular embodiment, when the pGenome and/or helper plasmid are modified as disclosed above in an alternative embodiment, the host cell may be modified to complement the expression of deleted or missing genes.
- 30

Rescued negative RNA virus

The present invention also provides a rescued negative RNA virus isolated from the eukaryotic cell transformed according to the method as defined above, preferably after 1 day post-transfection, more preferably after 3 days post-transfection.

5

Helper plasmid

The present invention also provides a helper plasmid pNPL comprising at least the sequences of the structural viral proteins of a virus belonging to the *Paramyxoviridae* family, in particular the Newcastle Disease Virus (NDV) or Peste des Petits Ruminants Virus (PPRV), consisting of nucleocapsid protein (N), phosphoprotein (P) and large protein (L), under control of a promoter, in particular a CMV promoter.

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In a particular embodiment, the helper plasmid pNPL of NDV comprises sequences SEQ ID N0:4.

In a particular embodiment, the pNPL plasmid of NDV comprises three independent expression cassettes under a promoter, in particular pCMV promoter to express N, P, L.

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In a particular embodiment, the helper plasmid pNPL of PPRV comprises sequence SEQ ID N0:29.

In a particular embodiment, the pNPL plasmid of PPRV comprises three independent expression cassettes under a promoter, in particular pCMV promoter to express N, P, L.

20

Kit

Another aspect of the present invention relates to a kit comprising, in one or more containers in a single package, a pGenome plasmide and a pNPL helper plasmid as defined above.

Particular kits according to the present invention further comprise a means for communicating information or instructions, to help using the kits' elements.

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In a particular embodiment, a kit for *in vitro* direct rescue of negative RNA virus from low virulent virus strain or lentogenic-like virus strain according to the invention comprises at least:

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- (i) a pGenome plasmid comprising at least a sequence encoding the virus genome from low virulent virus strain(s) or lentogenic-like virus strain(s), preferably belonging to the *Paramyxoviridae* family, in particular the Newcastle Disease Virus (NDV) or Peste des Petits Ruminants Virus (PPRV), and/or
- (ii) a pNPL helper plasmid comprising at least the sequences of the structural viral

proteins of a negative RNA virus, preferably belonging to the *Paramyxoviridae* family, in particular the Newcastle Disease Virus (NDV) or Peste des Petits Ruminants Virus (PPRV), consisting of nucleocapsid protein (N), phosphoprotein (P) and large protein (L),

- 5 (iii) optionally host cells,
- (iv) optionally culture medium for transfecting and culturing host cells, and
- (v) optionally well-plates.

In a preferred embodiment, a kit for *in vitro* direct rescue of negative RNA virus from low virulent virus strain or lentogenic-like virus strain according to the invention comprises at least:

- (i) a pGenome plasmid comprising at least a sequence encoding the virus genome from low virulent virus strain(s) or lentogenic-like virus strain(s) of the Newcastle Disease Virus (NDV) or Peste des Petits Ruminants Virus (PPRV), and/or
- 15 (ii) a pNPL helper plasmid comprising at least the sequences of the structural viral proteins of the Newcastle Disease Virus (NDV) or Peste des Petits Ruminants Virus (PPRV), consisting of nucleocapsid protein (N), phosphoprotein (P) and large protein (L),
- (iii) optionally host cells,
- 20 (iv) optionally culture medium for transfecting and culturing host cells, and
- (v) optionally well-plates.

In an alternative embodiment according to the invention for the kit:

- a) either the pGenome plasmid comprises a sequence encoding a virus genome which is partially deleted or mutated, and the host cell is optionally modified for expressing the said deleted gene(s),
- 25 b) either the pNPL helper plasmid is substituted by a helper plasmid comprising one or two sequences selected from the group consisting of sequences encoding N, P and L proteins, and the host cell is modified to complement the expression of missing
- 30 protein(s).

In a particular and preferred embodiment, the pGenome and pNPL helper plasmid are provided in the same kit.

In another particular embodiment, the pGenome and pNPL helper plasmid are provided in separated kits.

In conclusion, the present invention provides an improved reverse genetics system for negative RNA viruses allowing increased rescue of low virulent or attenuated virus.

Other characteristics and advantages of the invention appear in the following description of non-limitative illustrated figures and examples.

DESCRIPTION OF THE FIGURES

Figure 1. Plasmid constructions made in this study. (A, B and C) pN, pP and pL plasmids include N, P and L gene from MG-725 strain, respectively. (D) In the pNPL plasmid, three expression cassettes consisting of pCMV and polyA tail flanking the N, P or L gene from MG-725 were cloned into the same pCMV vector engineered from a pKS plasmid (see Material and Methods). (E) Downstream, the pMini-genome plasmid includes the promoter pCMV, the leader and trailer of MG-725 flanking the EGFP gene placed in antisense direction. (F) Complete genomes of NDV were assembled by RT-PCR and restriction enzymes into pCMV vector, between the two ribozymes.

Figure 2. Mini-genome assay with the 4-plasmids system. (A) Fluorescence appeared after transfection of BHK21 with the mini-genome expressing EGFP and pN, pP and pL. (B) Fluorescence did not appear when pL was not included in the plasmid cocktail used for transfection. Pictures were taken two days after transfection at 10× magnification. (C) Optimization of the 4-plasmids system. Histograms show the average and bars are the SD of the number of cells expressing EGFP in triplicate wells(s) of 24-well plates in the same assay.

Figure 3. Alignment of F protein cleavage sites from the rescued strains in this study and in other strains retrieved from GenBank (SEQ ID N0:19 to SEQ ID N0:25). Multiple alignments performed done using the Clustal W method in the DNA star software. The different amino acids in the low-virulent, virulent and rescued strains in the region encompassing residues 110-120 are framed. The vertical arrow indicates the trypsin cleavage site.

Figure 4. Rescue efficiency of the 4-plasmids system. (A) Three days after transfection, viruses were recovered and passaged in eggs for three days then tested by qRT-PCR as detailed in 'Materials and Methods'. The pMG-725/Fmu plasmid and H₂O were used as positive and negative controls of the qRT-PCR. Only two attenuated strains (LaSota/M Fmu and LaSota/Cherry) were not rescued. (B) Allantoic fluids showing HA and qRT-PCR positive results were considered as successfully rescued (V and L stand for strains with velogenic-like and lentogenic-like F protein cleavage site, respectively). All three velogenic strains (100%) were rescued, versus 6 out of 8 lentogenic strains (75%). (C to F) Three days after transfection with pMG-725/EGFP (C), pMG-725/Cherry (D), pMG-725/Fmu/EGFP (E) and pMG-725/Fmu/Cherry (F), cells were observed under a fluorescent microscope at magnification 10×. The two velogenic strains (C and D) show a clear enhancement of the green-fluorescent cells compared to the lentogenic strains (E and F).

Figure 5. Optimization of the 2-plasmids system on EGFP mini-genome assay and comparison with the 4-plasmids assay. (A) The 2-plasmids system was optimized by changing the quantitative ratio of pMini-genome and pNPL from 9:1 to 1:9 as shown on the X-axis. EGFP positive cells were then enumerated under a fluorescent microscope at magnification 5×, in 10 fields of one well of 6-well plates. (B and C) Comparative EGFP mini-genome performance between the 4- and 2-plasmids systems. The pictures were taken 2 days after transfection and suggest a higher number of fluorescent cells with the 2-plasmids systems (Magnification, 5×). (D) The improved performance was quantified by enumerating EGFP positive cells in 10 fields at magnification 5× of one well of 6-well plates. Data show the average and SD of triplicate wells

Figure 6. Comparative performance of 2- and 4-plasmids systems in rescuing one fluorescent lentogenic-like NDV strain. (A and B) BHK-21 cells were transfected with pMG-725/Fmu/EGFP (feature of lentogenic strain) and either pN, pP and pL (A) or pNPL (B). After three days, pictures were taken under the fluorescent microscope at magnification 20×. The 2-plasmids system shows a higher number of cells expressing EGFP. (C) The resulting viral titers were then determined on chicken embryos. The histograms show a 4.5-fold increase in the titer with the 2-plasmids system after standardization of the titer achieved with the 4-plasmids system. (D to G). For the evaluation of the limiting conditions for the 2-plasmids system, the quantities of the plasmid cocktail pMG-725/Fmu/EGFP and pNPL (quantitative

ratio 1:1) ranged from 1 to 4 µg, tested in virus rescue assays. Three days after transfection, pictures were taken under the fluorescent microscope at magnification 10×. Rescue was successful with as little as 2 µg of plasmids.

- 5 **Figure 7.** Efficiency of the 2- and 4-plasmids system on 9 NDV strains. (A and B) The rescue of two velogenic-like (V) and seven lentogenic-like (L) strains was tested in the two systems. The 2-plasmids system outperformed the 4-plasmids system under the limiting conditions of use consisting of 2 µg plasmids for transfection and virus recovery 1 day after transfection (A). Under the conditions of 3 µg of plasmids and recovery 3 days after transfection, the 2-
10 plasmids system performed better than the other for the lentogenic strains. In addition, under these conditions, more fluorescent cells were generated after transfection pMG-725/EGFP (C, G), pMG-725/Fmu/EGFP (D, H), pMG-725/Fmu/Cherry (E, I) and pLaSota/Cherry (F, J), with the 2-plasmids system (G to J) than with the 4-plasmids system (C to F). The pictures were taken at magnification 10×.

15

EXAMPLES

Materials and Methods

- Cells and Viruses.** Baby hamster kidney BHK-21 cells were grown in Eagle's minimum
20 essential medium (Gibco) with 10% fetal bovine serum (PAN-Biotch) and cultured at 37 °C with 5% CO₂. Chemically competent cells, 10-beta strains, were purchased from New England Biolabs (NEB). NDV chicken/Madagascar/2008 (MG-725) strain, isolated from chicken in Madagascar (19), was amplified in 10 day-old specific pathogen free (SPF) chicken embryos (Couvoir de Cerveloup, France). After two days of infection, allantoic liquid
25 was harvested and stored at -80 °C. NDV LaSota strain (Genbank accession numbers AY845400.2, AF077761 or JF950510, 15186 bp linear RNA, SEQ ID N0: 12), kindly provided by ISZVe, Italy, was amplified and stored in the same way as the MG-725 strain.

- PPRV from Nigeria 75/1 attenuated vaccine strain was isolated originally from a sick goat on primary lamb kidney cell culture in Nigeria and attenuated by serial passages on Vero
30 cells (Diallo et al., 1989) (Genbank accession number KY628761.1 or X74443.2, 15948 bp linear RNA, SEQ ID N0: 31).

Plasmid constructions. RNA of MG-725 was extracted using the NucleoSpin RNA virus kit (MACHEREY-NAGEL) according to the manufacturer's instructions. RNA was used to

generate cDNA with the Super Script III First-Stand kit (Invitrogen). Pfu Ultra Fusion HS DNA polymerase (Agilent) was used to amplify the N, P and L genes of MG-725 from cDNA and the three genes were cloned into the pCI-neo plasmid by restriction enzymes (NEB) (see Table 1, Fig.1A to C).

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TABLE 1. Information on all plasmids used in the present invention. The first four were constructed into the pCI-neo plasmid (Promega) while the rest were built into the pBluescript II SK(+/-) plasmid (Stratagene)

SEQ ID NO:	Plasmids	Backbone of complete genome or gene	Properties	F protein cleavage sites
1	pN	MG-725	N gene of MG-725	-
2	pP	MG-725	P gene of MG-725	-
3	pL	MG-725	L gene of MG-725	-
4	pNPL	MG-725	N,P and L genes of MG-725	-
5	pMini-genome	MG-725	Leader and trailer of MG-725, EGFP from pEGFP-C1	-
6	pMG-725	MG-725	Complete genome of MG-725	Velogenic-like ^a
7	pMG-725/EGFP	MG-725	EGFP gene was inserted between P and M genes of the complete MG-725 genome	Velogenic-like
8	pMG-725/Cherry	MG-725	mCherry gene was inserted between P and M genes of the complete MG-725 genome	Velogenic-like
9	pMG-725/Fmu	MG-725	Cleavage site of the F protein in MG-725 was modified to that of LaSota	Lentogenic-like ^b
10	pMG-725/Fmu/EGFP	MG-725	Cleavage site of the F protein in MG-725 /EGFP was modified to that of LaSota.	Lentogenic-like
11	pMG-	MG-725	Cleavage site of the F	Lentogenic-

	725/Fmu/Cherry		protein in MG-725 /Cherry was modified to that of LaSota.	like
12	pLaSota	LaSota	The complete genome of LaSota	Lentogenic-like
13	pLaSota/Cherry	LaSota	Cherry gene was inserted between P and M genes of LaSota	Lentogenic-like
14	pLaSota/M-Fmu	LaSota	The F gene of LaSota was replaced by that of MG-725/Fmu	Lentogenic-like
15	pLaSota/M-HN	LaSota	The HN gene of LaSota was replaced by that of MG-725	Lentogenic-like
16	F259 primer		F259 forward primer for qRT-PCR	
17	F488 primer		F488 reverse primer for qRT-PCR	
18	GRQGRL		modified F protein cleavage site	
19	GGGRQGRLIGA		F protein cleavage site from low virulent strains B1, LaSota and HX01 (Figure 3)	
20	GGRRQRRFIGA		F protein cleavage site from virulent strain F48E9 (Figure 3)	
21	GGRRQKRFIGA		F protein cleavage site from virulent strain ZJ1 (Figure 3)	
22	GGRRRRRFVGA		F protein cleavage site from virulent strain MG-1992 or rescued rMG-	

				725 strains (Figure 3)	
23		GGGRQGRLVGA		F protein cleavage site from rescued rMG-725 strains (Figure 3)	
24		GGGRQGRLIGA		F protein cleavage site from rescued rLaSota strains (Figure 3)	5
25		GGGRQGRLVGA		F protein cleavage site from rescued rLaSota strains (Figure 3)	10
26		pN	Nigeria 75/1	N gene of PPR Nigeria 75/1 strain	
27		pP	Nigeria 75/1	P gene of PPR Nigeria 75/1 strain	
28		pL	Nigeria 75/1	L gene of PPR Nigeria 75/1 strain	15
29		pNPL	Nigeria 75/1	plasmid with N, P and L genes of PPR Nigeria 75/1 strain	
30		pCMV-PPRV-eGFP	Nigeria 75/1	plasmid including CMV promoter, the full genome of PPR vaccine strain Nigeria 75/1 and a cassette expressing eGFP	20 25
31		PPRV	Nigeria 75/1	Complete genome (Genbank accession number KY628761.1 or X74443.2)	30

^a F protein cleavage sites are ¹¹²RRRRRF¹¹⁷.

^b F protein cleavage sites are ¹¹²GRQGRL¹¹⁷.

The T7 RNA polymerase promoter and terminator of pKS plasmid were replaced by the CMV promoter and polyA from pCI-neo. The two ribozymes were then inserted between the CMV promoter and polyA sequences. A fragment with multiple cloning sites was introduced between the two ribozymes to obtain a pCMV vector. Then, the N, P and L genes of the MG-725 strain flanked by the CMV promoter and polyA tail were amplified from pN, pP and pL and cloned into the same pCMV vector to generate pNPL plasmid (Fig. 1D).

A mini-genome plasmid was also prepared to assess the usefulness of the helper plasmids. In this plasmid, the enhanced green fluorescence protein (EGFP) gene was flanked by the leader and trailer strains of MG-725 by overlap PCR and then cloned into pCMV vector, between ribozymes, in the reverse direction to get the pMini-genome (Fig. 1E).

The complete MG-725 genome was divided into eight fragments with overlap regions. These fragments were amplified from viral RNA and were assembled into pCMV vector, between two ribozymes, to generate pMG-725 plasmid according to the order of virus genome (Leader-N-P-M-F-HN-L-Trailer) and restriction sites (Fig. 1F). F gene of MG-725 was modified by overlap PCR to obtain a lentogenic cleavage site identical to that of the LaSota strain (hereafter called Fmu). The Fmu gene replaced that of pMG-725 plasmid to obtain pMG-725/Fmu. The EGFP or mcherry fluorescent gene with gene start (GS) and gene end (GE) of MG-725 was amplified from pEGFP-C1 or pmCherry-N1 (Clontech) and then cloned into pMG-725 and pMG-725/Fmu plasmids, between the P and M genes, to get pMG-725/EGFP, pMG-725/Cherry, pMG-725/Fmu/EGFP, and pMG-725/Fmu/Cherry plasmids.

The complete genome of the LaSota strain was divided into seven fragments. These seven fragments were amplified and assembled into pCMV to get pLaSota. The mcherry fluorescent gene with GS and GE of LaSota was inserted between the P and M genes of pLaSota to generate pLaSota/Cherry. The F and HN genes of pLaSota were replaced individually with those of pMG-725/Fmu to get pLaSota/M-Fmu, pLaSota/M-HN. All these plasmids were purified with the Quick Plasmid Miniprep kit (Invitrogen) and the EndoFree plasmid Maxi kit (QIAGEN), aliquoted and stored at -20 °C. The total number of plasmids produced with the complete viral genome was 10 (Table 1).

Similar constructions have been obtained with PPRV from Nigeria 75/1 attenuated vaccine strain isolated originally from a sick goat on primary lamb kidney cell culture in Nigeria and attenuated by serial passages on Vero cells (Diallo et al., 1989).

Example 1: Validation of 4-plasmids and 2-plasmids systems on the EGFP mini-genome.

4×10⁵ BHK-21 cells were seeded in 6-well plates one day before transfection. A total of 5
5 µg of plasmids were transfected into the cells using Lipofectamine 2000 (Invitrogen). Different
plasmid cocktails were tested: [pMini-genome, pN, pP and pL, ratio 5:2:2:1], [pMini-genome,
pN, pP and pCl-neo, ratio 5:2:2:1], [pMini-genome and pNPL, ratio, 1:1] and [pMini-genome
and pCl-neo, ratio, 1:1]. After six hours, the transfection media were replaced by 2 ml of MEM
medium containing 10% FBS. Cells expressing EGFP were checked daily for 3 days after
10 transfection. EGFP positive cells were enumerated in 10 different fields under the fluorescent
microscope 2 days after transfection. To optimize the 4-plasmids system, 1×10⁵ BHK-21 cells
were seeded on 24-well plates one day before transfection. A mixture of 0.5 µg pMini-
genome, 0.1 µg pL and 0.4 µg of [pN and pP, with ratios ranging from 9:1 to 1:9] was then
transfected into cells. The number of cells expressing EGFP was determined under the
15 fluorescent microscope two days after transfection. To optimize the 2-plasmids system, 2.5
µg of pMini-genome and pNPL with ratios ranging from 9:1 to 1:9 were transfected into BHK-
21 cells seeded on 6-well plates. EGFP positive cells were enumerated in 10 fields under the
microscope two days after transfection.

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Example 2: Virus rescue with the 4-plasmids system.

4×10⁵ BHK-21 cells were seeded on 6-well plates the day before transfection. Each of the
10 complete viral genome plasmids (Table 1) was mixed with three other plasmids, pN, pP
and pL, to obtain a total quantity of 10 µg with a ratio of 5:2:2:1 [pGenome: pN: pP: pL].
25 These mixtures were transfected into the cells and three days later, images were taken for
fluorescent viruses and cell supernatants were collected. Attached cells were removed by
incubation in presence of trypsin, centrifuged for 5 min at 1,000 rpm and suspended again in
200 µL of the previously collected media. These cell samples were injected into 10 day old
SPF chicken embryos for virus amplification.

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Example 3: Generation of the G-725/Fmu/EGFP strain with the 4-plasmids and the 2-plasmids systems.

Based on the results obtained using the method described in the previous section, we
decided to develop an improved reverse genetics system based on the transfection of only

two plasmids. We then compared the rescue viral titers of the 4-plasmids and 2-plasmids systems. To this end, either [5 µg of pMG-725/Fmu/EGFP, 2 µg pN, 2 µg pP and 1 µg pL] or [5 µg pMG-725/Fmu/EGFP and 5 µg pNPL] were transfected into cells seeded in a 6-well plate. Three days after transfection, the EGFP expression of cells was checked. Next, cell supernatants were collected and diluted 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 times in MEM. 200 µL of these dilutions were injected into chicken embryos for virus titration.

To test the quantity of plasmids and the time taken by the 2-plasmids system rescue virus, the different amounts of pMG-725/Fmu/EGFP and pNPL to be transfected into BHK-21 cells collected at different times were assessed. The total amount of these plasmids ranged from 1 µg to 10 µg with a ratio of 1:1. The cells with 200 µl of their supernatants were collected three days after transfection, except the cells transfected with 10 µg of plasmids, which were collected one, two and three days after transfection with 200 µl of their supernatant. These samples were injected into chicken embryos.

Example 4: Comparison of the two systems with a set of complete genomes including virulent and attenuated phenotypes.

Nine different complete genomes comprising two virulent phenotypes (pMG-725, pMG-725/EGFP) and seven attenuated strains (pMG-725/Fmu, pMG-725/Fmu/EGFP, pMG-725/Fmu/Cherry, pLaSota, pLaSota/Cherry, pLaSota/M-Fmu or pLaSota/M-HN) were transfected with either pN, pP and pL or pNPL, into BHK-21 cells plated on 6-well plates. Based on the results obtained in the previous section, the total amount of the plasmids delivered to the cells was set at 2 and 3 µg with a ratio of 5:2:2:1 [pGenome: pN: pP: pL] or 1:1 [pGenome: pNPL]. As described in the previous section, one or three days after transfection, fluorescence was checked under the microscope and cells and their supernatants were collected and used for virus amplification in eggs.

4.1 Confirmation of rescued virus.

Dead chicken embryos and chicken embryos that were still alive three days after inoculation at 37 °C were left overnight at 4 °C. Allantoic fluids were collected and used to perform a hemagglutination assay (HA) and a real-time PCR (qRT-PCR). After extraction, RNAs were first digested with TURBO DNase enzyme (Ambion) to prevent DNA contamination. The qRT-PCR used F259 (5'-ACAYTGACYACTTTGCTCA-3'= SEQ ID N0:16) as forward primer and F488 (5'-TGACAGCYTCATTGGTTGC-3'= SEQ ID N0:17) as reverse primer. These

primers were designed by us based on the alignment of different strains' F genes and matched the F NDV gene. The Brilliant III Ultra-Fast SYBR Green QRT-PCR Master mix kit (Agilent) was used. Only samples showing positive results in both HA and qRT-PCR were considered successfully rescued. Negative allantoic fluids were passaged twice in chicken embryos and tested again by HA and qRT-PCR.

4.2 The rescue performance of the 4-plasmids system was lower in the case of attenuated NDV strains.

After transfection of BHK21 with the mini-genome expressing EGFP in the 4-plasmids system, green fluorescence was observed in the cells (Fig. 2A). As expected, this fluorescence was not seen in the absence of pL (Fig. 2B). The pMini-genome and pL were transfected into BHK-21 cells with different quantity ratios for pN and pP to achieve the best performance. The best quantity ratio for pN and pP was 1:1 (Fig. 2C).

This optimized 4-plasmids system was then used to rescue 10 different viruses, three of which were expected to be velogenic (virulent), while the seven others were lentogenic (attenuated) (Table 1). This distinction is based on the amino acid motif found at the F protein cleavage site. The velogenic strains have five basic amino acids, while the lentogenic strains have two basic amino acids (Fig. 3). This difference makes the F protein of virulent strains more prone to be cleaved by various proteases present in various tissues and the virus is then activated to amplify whereas the F protein of attenuated strains is only cleaved in environments like the digestive and respiratory tracts or *in vitro*, in cell culture medium containing trypsin (13). Using the 4-plasmids system, strains with a velogenic-like cleavage site were clearly more efficiently rescued than strains with the same backbone only differing by a lentogenic-like cleavage site (Fig. 4 C to F). In addition, two lentogenic strains out of the 10 strains could not be rescued after inoculation of the egg, additional blind passages in eggs and HA test and qRT-PCR (Fig. 4A, Table 2). This resulted in a rescue efficiency of only 71% with attenuated strains, whereas for velogenic viruses, the efficiency was 100% (Fig. 4B). These results suggest that the 4-plasmids reverse genetics system is less efficient for the rescue of viruses with lentogenic-like F protein cleavage sites.

TABLE 2 Viruses used in the 4-plasmids system

Viruses	Rescuing
	^a

MG-725	+ ^b
MG-725/EGFP	+
MG-725/Cherry	+
MG-725/Fmu	+
MG-725/Fmu/EGFP	+
MG-725/Fmu/Cherry	+
LaSota	+
LaSota/Cherry	- ^c
LaSota/M-Fmu	-
LaSota/M-HN	+

^a The condition for the rescue of the virus was 10µg plasmids, 3 days after transfection.

^b Viruses were successfully rescued after confirmation by HA and qRT-PCR.

^c Viruses were not rescued.

5 **4.3 The 2-plasmids system outperformed the 4-plasmids system in the replication of the EGFP mini-genome.**

To test the 2-plasmids system, another plasmid, pNPL, was produced in which the N, P and L genes are inserted downstream from the CMV promoters (Fig.1E). As expected, cells transfected with pMini-genome and pNPL showed EGFP expression (Fig. 5C). In addition, the 2-plasmids system was the most efficient with a pMini-genome:pNPL ratio of 1:1 (Fig.5A). In comparison to the 4-plasmids system, the 2-plasmids system showed double the number of cells expressing the EGFP (Fig.5B to D). Based on EGFP mini-genome testing, these findings suggest that the 2-plasmids reverse genetics system is more effective than the 4-plasmids system.

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4.4 The 2-plasmids system generates more viruses with lentogenic-like F protein cleavage sites.

To test whether a 2-plasmids system would circumvent the reduced rescuing efficiency observed with our lentogenic viruses, pMG-725/Fmu/EGFP plasmid containing the full genome of an attenuated green fluorescent recombinant virus was transfected into BHK-21

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cells either with pN, pP and pL or pNPL. Based on the EGFP expression of transfected cells, the 2-plasmids system clearly outperformed the 4-plasmids system for the rescue of this lentogenic virus (Fig. 6A and B). In addition, the supernatant of transfected cells using the 2-plasmids system had a viral titer about 4.5 times higher than the titer achieved by the 4-plasmids system (Fig. 6C). These results demonstrate that the 2-plasmids system is more suitable for the rescue of lentogenic viruses.

In order to identify the conditions that limit the efficacy of the 2-plasmids system, rescue of the MG-725/Fmu/EGFP strain was tested at different plasmid concentrations for transfection and at different intervals after transfection for virus recovery. It was possible to reduce the total quantity of plasmids to be delivered to the cells with the 2-plasmids system to 2 µg and still successfully rescue the virus three days after transfection (Fig. 6D to G, Table 3). Rescue was also possible as soon as 24 h after cell transfection when 10 µg of plasmids was used for transfection (Table 3). These results indicate that rescuing a virus with only 2 plasmids can still be effective and rapid even with a small quantity of plasmids.

TABLE 3. Optimization of the 2-plasmids system on the rescue of the MG-725/Fmu/EGFP strain

	Rescue with 10 µg of plasmids and recovery 1-3 days after transfection			Rescue with 1 to 10 µg of plasmids and recovery 3 days after transfection									
Virus	Day 1	Day 2	Day 3	1	2	3	4	5	6	7	8	9	10
MG-725/Fmu/EGFP	+ ^a	+	+	- ^b	+	+	+	+	+	+	+	+	+

^a Viruses were considered as successfully rescued after confirmation by HA and qRT-PCR.

^b Viruses were not rescued.

4.5 The 2-plasmids system performs better than the 4-plasmids system under two different conditions.

To confirm the superiority of the 2-plasmids system, different viruses were rescued under two different limiting conditions. Under the first condition (2 µg plasmids, collection one day after

transfection), the 4-plasmids system successfully rescued only one velogenic virus (MG-725) from 9 complete genome plasmids (Table 4). In contrast, the 2-plasmids system was able to rescue five viruses, including three lentogenic viruses (Fig. 7A, Table 4). Under the second condition (3 µg plasmids, three days after transfection), the rescue efficiency increased to 67% for the 4-plasmids system, but three viruses with lentogenic-like F protein cleavage sites were still not rescued. In contrast, rescue efficiency was 100% with the 2-plasmids system (Fig. 7B, Table 4). Moreover, three days after transfection, the titers of both velogenic and lentogenic viruses were higher in the 2-plasmids system than in the 4-plasmids system, as evidenced by the number of cells expressing the EGFP or mcherry protein (Fig. 7C to 7J). These results demonstrate the better performance of the 2-plamid system whatever the conditions of use and the virulence of rescued viruses.

TABLE 4. Rescue efficiencies of 4- and 2-plasmids systems under two conditions on a set of 10 different viruses.

Viruses	Rescuing with:			
	4-plasmids		2-plasmids	
	A ^a	B ^b	A	B
MG-725	+ ^c	+	+	+
MG-725/EGFP	- ^d	+	+	+
MG-725/Fmu	-	+	+	+
MG-725/Fmu/EGFP	-	-	+	+
MG-725/Fmu/Cherry	-	+	+	+
LaSota	-	+	-	+
LaSota/Cherry	-	-	-	+
LaSota/M-Fmu	-	-	-	+
LaSota/M-HN	-	+	-	+

^a The condition for the rescue was 2 µg plasmids, one day after transfection.

^b The condition for the rescue was 3 µg plasmids, 3 days after transfection.

^c Viruses were considered as successfully rescued after confirmation by HA and qRT-PCR.

^d Viruses were not rescued.

5 **Example 5: Comparison of the two plasmids systems for rescuing an attenuated Peste des Petits Ruminants Virus (PPRV)**

5.1 Method with 4-plasmids system

4x10⁵ CHS cells (Adombi et al 2011, J Virol) were seeded on 6-well culture plates one day
10 before transfection. Four plasmids were transfected:

- (i) plasmid including the full genome of PPR vaccine attenuated strain Nigeria 75/1 with a cassette expressing eGFP and flanked in 3' end by CMV promoter and ribozyme, and in 5' end by ribozyme and poly A tail (SEQ ID N0: 30);
- (ii) plasmid with N gene of PPR (SEQ ID N0: 26) flanked by CMV promoter and poly
15 A tail;
- (iii) plasmid with P gene of PPR (SEQ ID N0: 27) flanked by CMV promoter and poly A tail; and
- (iv) plasmid with L gene of PPR (SEQ ID N0: 28) flanked by CMV promoter and poly
20 A tail.

Transfection was performed with lipofectamin 3000 with a final quantity of 2.5 µg of plasmid in proportion 5:2:2:1 [pGenome: pN: pP: pL]. Cells were put in contact with lipofectamin and plasmid (concentration following manufacturer's instruction) during 4h before removing lipofectamin mix and adding culture medium to cells. After several days, once cells reached
25 confluence, they were put in contact with trypsin to detach them and transferred into 25cm³ flasks with fresh medium. At confluence, they were transferred again in 75cm³ flasks with fresh medium.

Cells were observed with inverted microscope for cytopathic effect, then for fluorescence with
30 fluorescence microscope. Cells were passed again in 75cm³ (1/4 of cells) if fluorescence or cytopathic effect was not observed. Once cell infection was confirmed, they went through 3 cycles of freezing and thawing before centrifugation and collection of supernatant containing the rescued virus.

5.2 Method with 2-plasmids system

Transfection method was identical as in 5.1 except that only two plasmids in 10:1 ratio were used :

- (i) plasmid including the full genome of PPR vaccine strain Nigeria 75/1 with a cassette expressing eGFP and flanked in 3' end by CMV promoter and ribozyme, and in 5' end by ribozyme and poly A tail (SEQ ID N0:30);
- (ii) unique helper plasmid with N, P and L genes, each flanked by CMV promoter and poly A tail (SEQ ID N0:29).

5.3 Results

Fluorescent PPRV was obtained only once out of 21 attempts in the classical method based on 4-plasmids, whereas fluorescent PPRV was obtained twice out of 3 attempts with the new method using the 2-plasmids system according to the invention.

These results demonstrated that the 2-plasmids system according to the invention has also a better efficiency for rescuing attenuated PPRV than the classical 4 plasmids system.

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CLAIMS

1. *In vitro* method of rescuing negative RNA virus from low virulent virus strain(s) or lentogenic-like virus strain(s) belonging to the order Mononegavirales including
5 *Bornaviridae*, *Filoviridae*, *Mymonaviridae*, *Nyamiviridae*, *Paramyxoviridae*, *Pneumoviridae*, and *Rhabdoviridae* families, preferably belonging to the *Paramyxoviridae* family, comprising at least the steps of:
 - (i) co-transfecting host cells with a two-plasmid system comprising
 - 10 a. a pGenome plasmid comprising at least a sequence encoding a virus genome from low virulent virus strain(s) or lentogenic-like virus strain(s) and
 - b. a pNPL helper plasmid comprising at least the sequences encoding the structural viral proteins nucleocapsid protein (N), phosphoprotein (P) and large protein (L),
 - 15 (ii) culturing host cells under conditions for replication and transcription of the virus, and
 - (iii) recovering the rescued negative strand RNA viruses.
2. *In vitro* method of rescuing negative RNA virus according to claim 1, wherein
20 alternatively:
 - a) either the pGenome plasmid comprises a sequence encoding a virus genome which is partially deleted or mutated, and the host cell is optionally modified for expressing the said deleted gene(s), and/or
 - 25 b) either the pNPL helper plasmid is substituted by a helper plasmid comprising one or two sequences selected from the group consisting of sequences encoding N, P and L proteins, and the host cell is modified to complement the expression of missing protein(s).
3. *In vitro* method of rescuing negative RNA virus according to claims 1 or 2, wherein the
30 negative RNA virus is a virus belonging to the *Paramyxoviridae* family, in particular the Newcastle Disease Virus (NDV) or Peste des Petits Ruminants Virus (PPRV).
4. *In vitro* method of rescuing negative RNA virus according to anyone of claims 1 to 3,

wherein the low virulent virus strain is selected from PPRV Nigeria 75/1 strain (SEQ ID N0:31) or PPRV recombinant Nigeria 75/1 strain.

5. *In vitro* method of rescuing negative RNA virus according to anyone of claims 1 to 3,
5 wherein the low virulent virus strain is selected from the group consisting of virus strains having a genotype with a lentogenic-like F protein cleavage site, in particular a sequence formula (I) $^{112}X_1-X_2-X_3-X_4-X_5-X_6^{117}$ wherein X_2 and X_5 are independently arginine (R) or lysine (K), preferably arginine (R), and X_1, X_3, X_4, X_6 are independently selected from the group consisting of non-basic amino acids.
- 10 6. *In vitro* method of rescuing negative RNA virus according to claim 5, wherein the low virulent virus strain is selected from NDV LaSota strain (SEQ ID N0:12) or NDV recombinant LaSota strain.
- 15 7. *In vitro* method of rescuing negative RNA virus according to anyone of claims 1 to 6, wherein the weight ratio between the first plasmid pGenome and the helper plasmid pNPL ranges from 9:1 to 1:9, and is preferably 1:1.
- 20 8. *In vitro* method of rescuing negative RNA virus according to anyone of claims 1 to 7, wherein the host cells are eukaryotic cells, in particular mammal cells, preferably baby hamster kidney cells (BHK-21).
- 25 9. *In vitro* method of rescuing negative RNA virus according to anyone of claims 1 to 8, wherein the host cells are transfected with an amount of two-plasmid system ranging from 1 μ g to 20 μ g, in particular from 2 to 20 μ g, and preferably from 3 to 5 μ g.
- 30 10. *In vitro* method of rescuing negative RNA virus according to anyone of claims 1 to 9, comprising additionally a step of amplification of RNA virus into chicken embryos between steps (ii) and step (iii).
- 35 11. A eukaryotic host cell transformed with the two-plasmid system according to the method as defined in anyone of claims 1 to 10.
12. A rescued negative RNA virus isolated from the eukaryotic cell transformed according to the method as defined in anyone of claims 1 to 10, preferably after 1 day post-

transfection, more preferably after 3 days post-transfection.

13. A helper plasmid pNPL comprising at least the sequences of the structural viral proteins of a virus belonging to the *Paramyxoviridae* family, in particular the Newcastle Disease Virus (NDV) or Peste des Petits Ruminants Virus (PPRV), consisting of nucleocapsid protein (N), phosphoprotein (P) and large protein (L), under control of a promoter, in particular a CMV promoter.
14. A kit for direct rescue of negative RNA virus from low virulent virus strain or lentogenic-like virus strain *in vitro* comprising at least:
- (i) a pGenome plasmid comprising at least a sequence encoding the virus genome, preferably belonging to the *Paramyxoviridae* family, in particular the Newcastle Disease Virus (NDV) or Peste des Petits Ruminants Virus (PPRV), and/or
 - (ii) a pNPL helper plasmid comprising at least the sequences of the structural viral proteins of a negative RNA virus, preferably belonging to the *Paramyxoviridae* family, in particular the Newcastle Disease Virus (NDV) or Peste des Petits Ruminants Virus (PPRV), consisting of nucleocapsid protein (N), phosphoprotein (P) and large protein (L),
 - (iii) optionally host cells,
 - (iv) optionally culture medium for transfecting and culturing host cells, and
 - (v) optionally well-plates.
15. A kit for direct rescue of negative RNA virus from low virulent virus strain or lentogenic-like virus strain according to claim 14, wherein alternatively :
- a) either the pGenome plasmid comprises a sequence encoding a virus genome which is partially deleted or mutated, and the host cell is optionally modified for expressing the said deleted and/or mutated gene(s), and/or
 - b) either the pNPL helper plasmid is substituted by a helper plasmid comprising one or two sequences selected from the group consisting of sequences encoding N, P and L proteins, and the host cell is modified to complement the expression of missing protein(s).

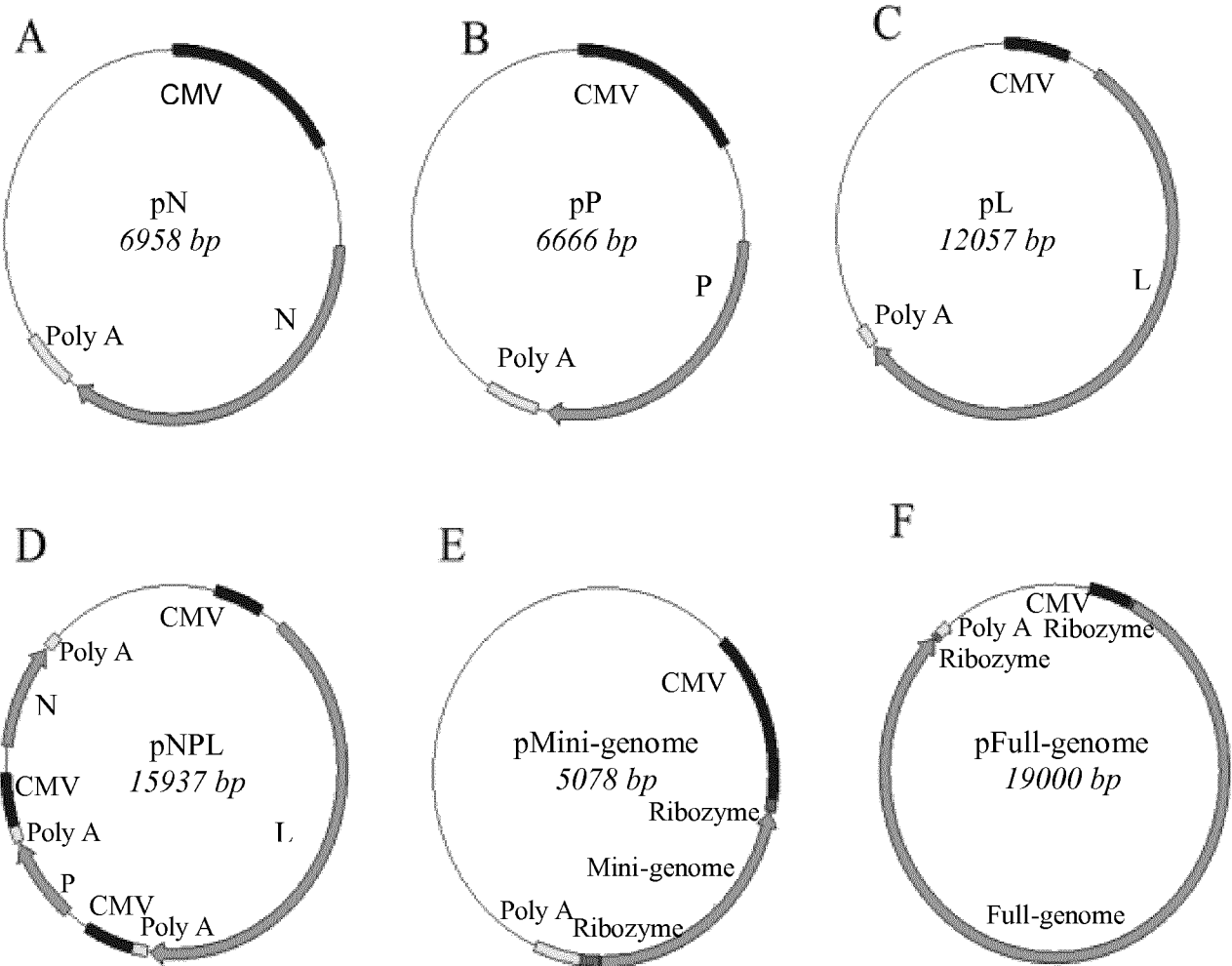


FIGURE 1

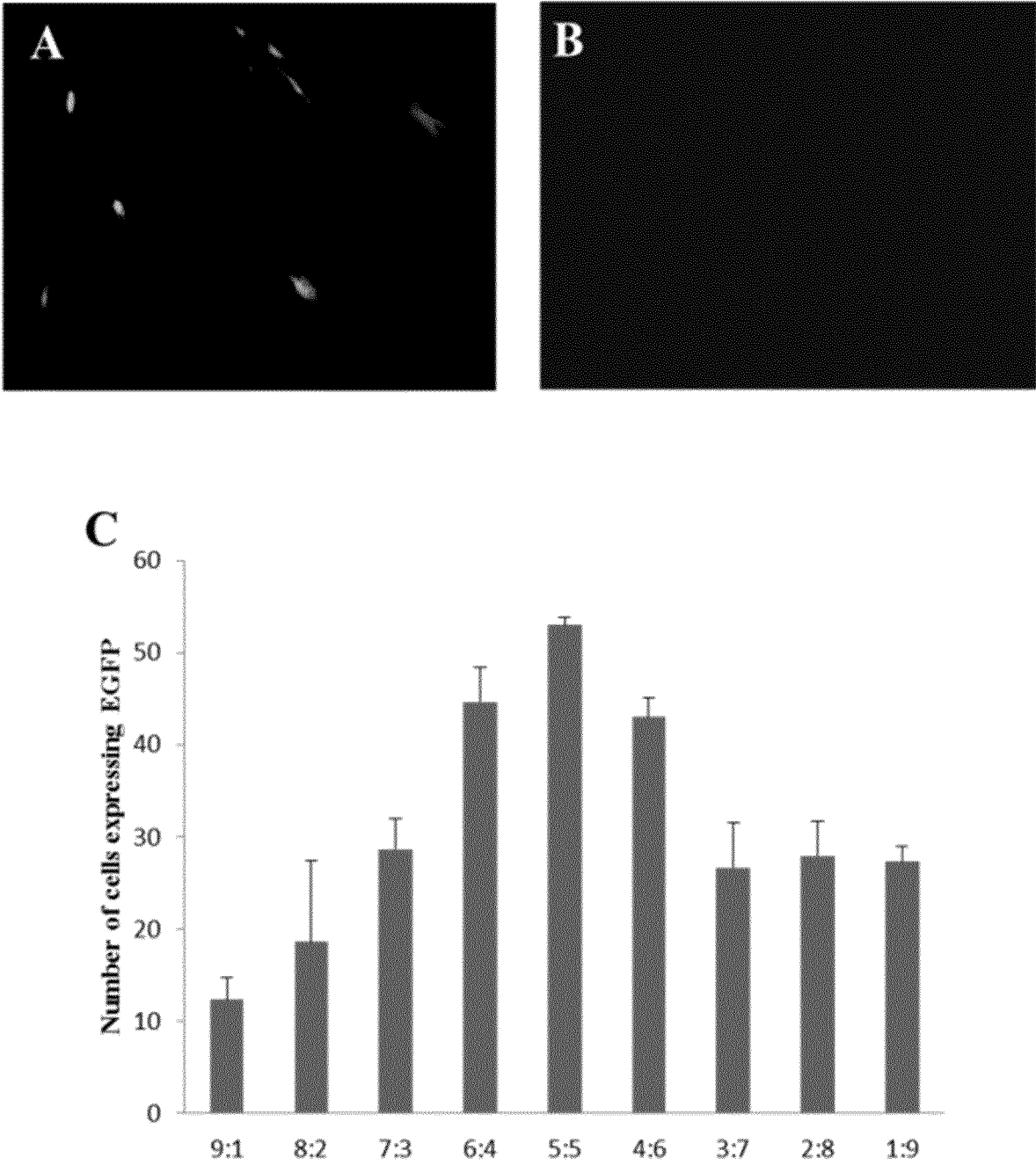


FIGURE 2

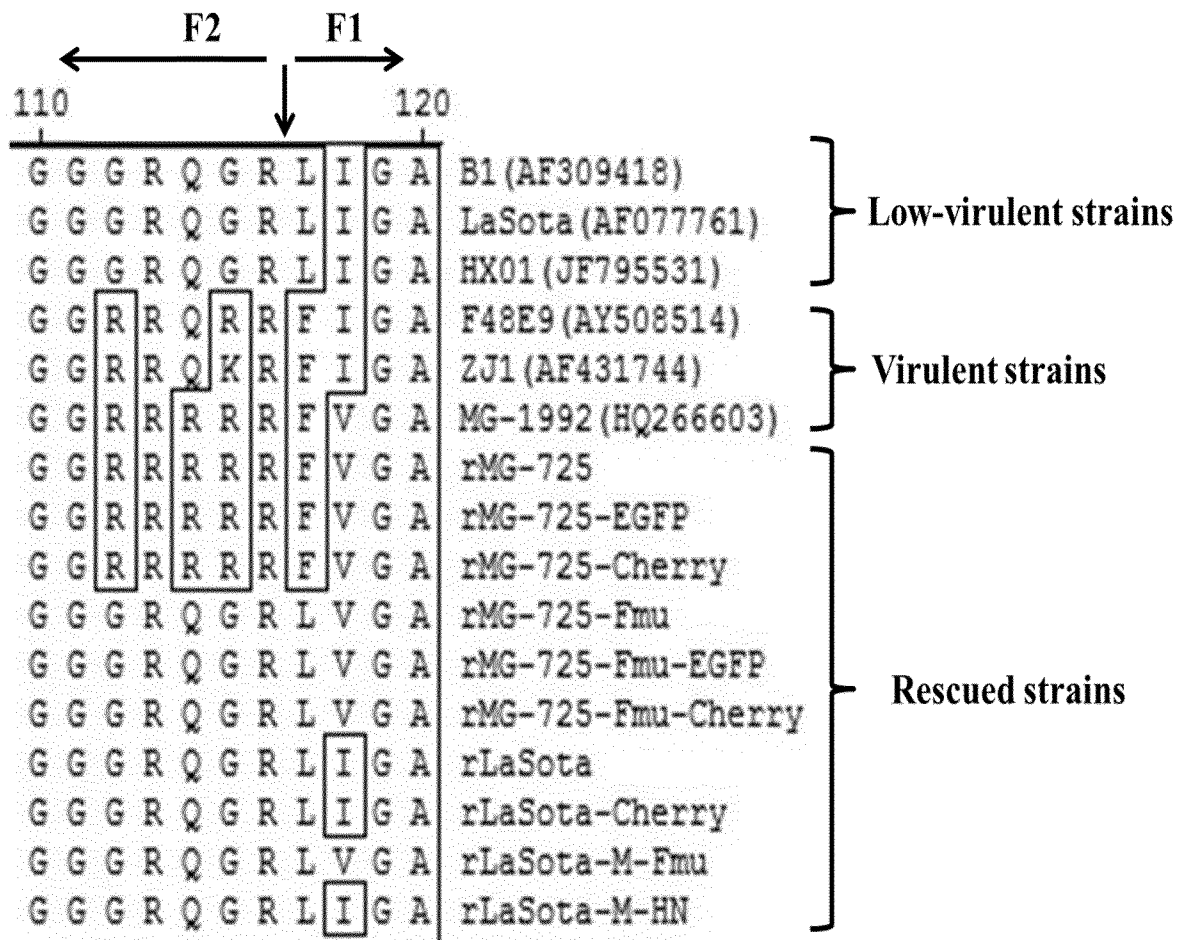


FIGURE 3

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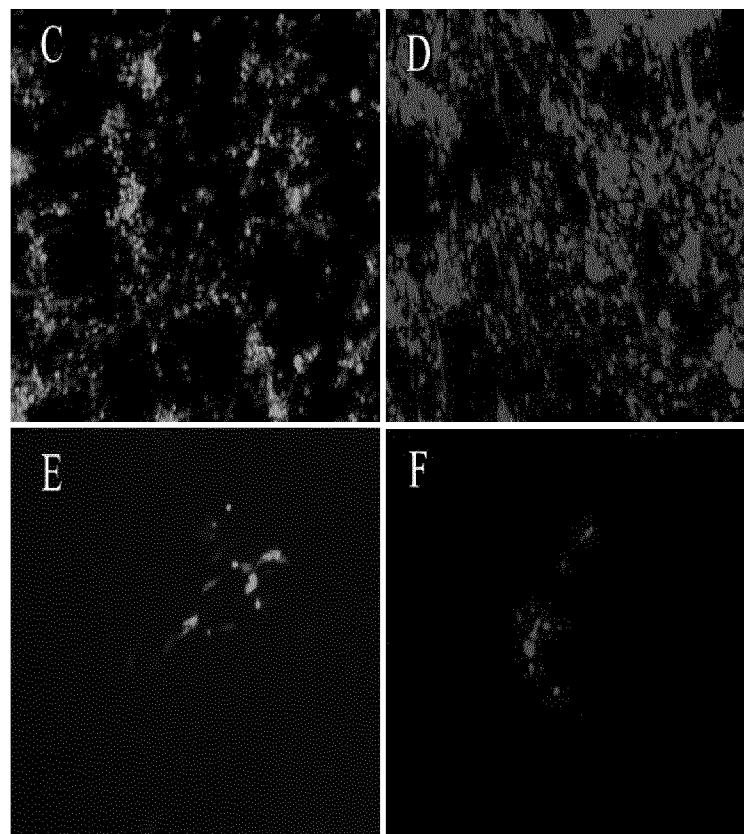
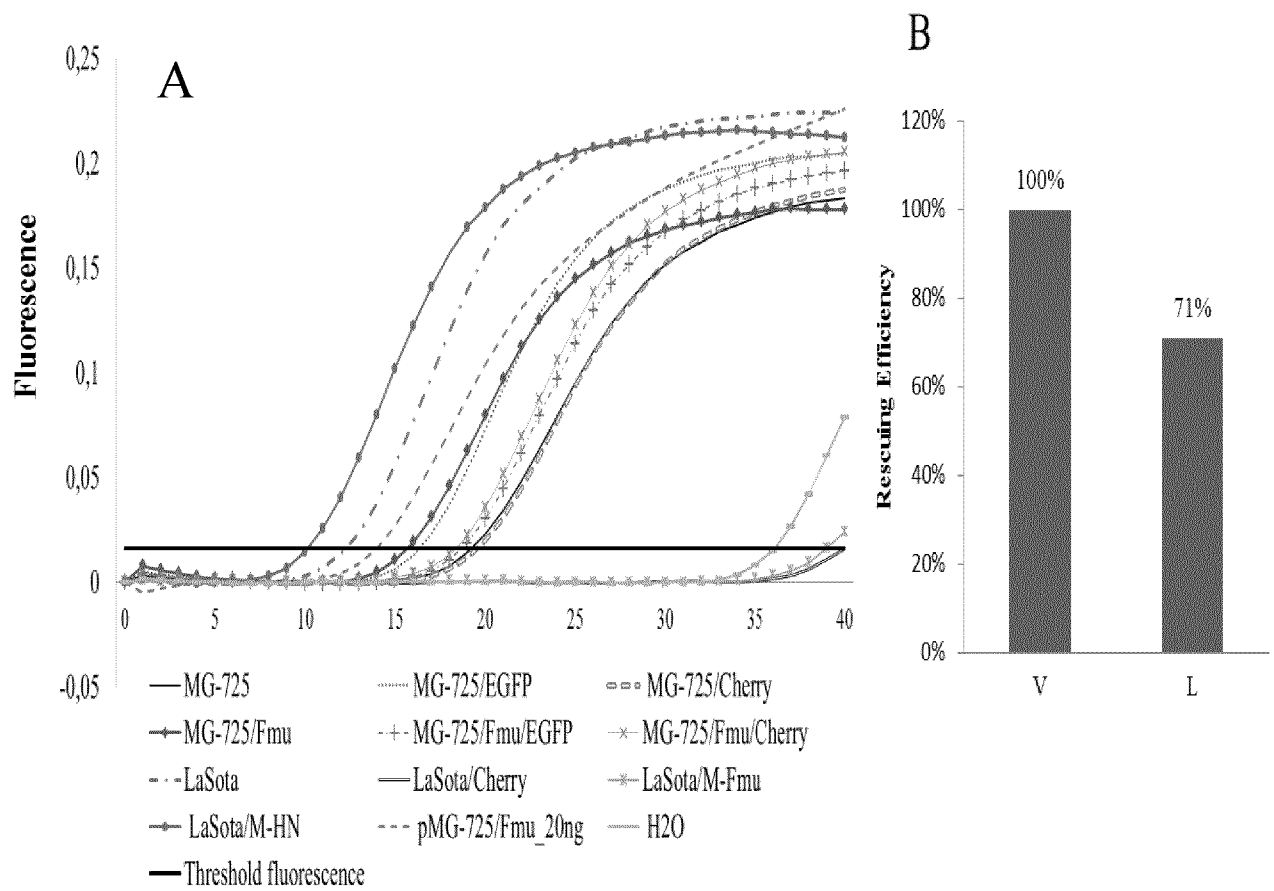


FIGURE 4

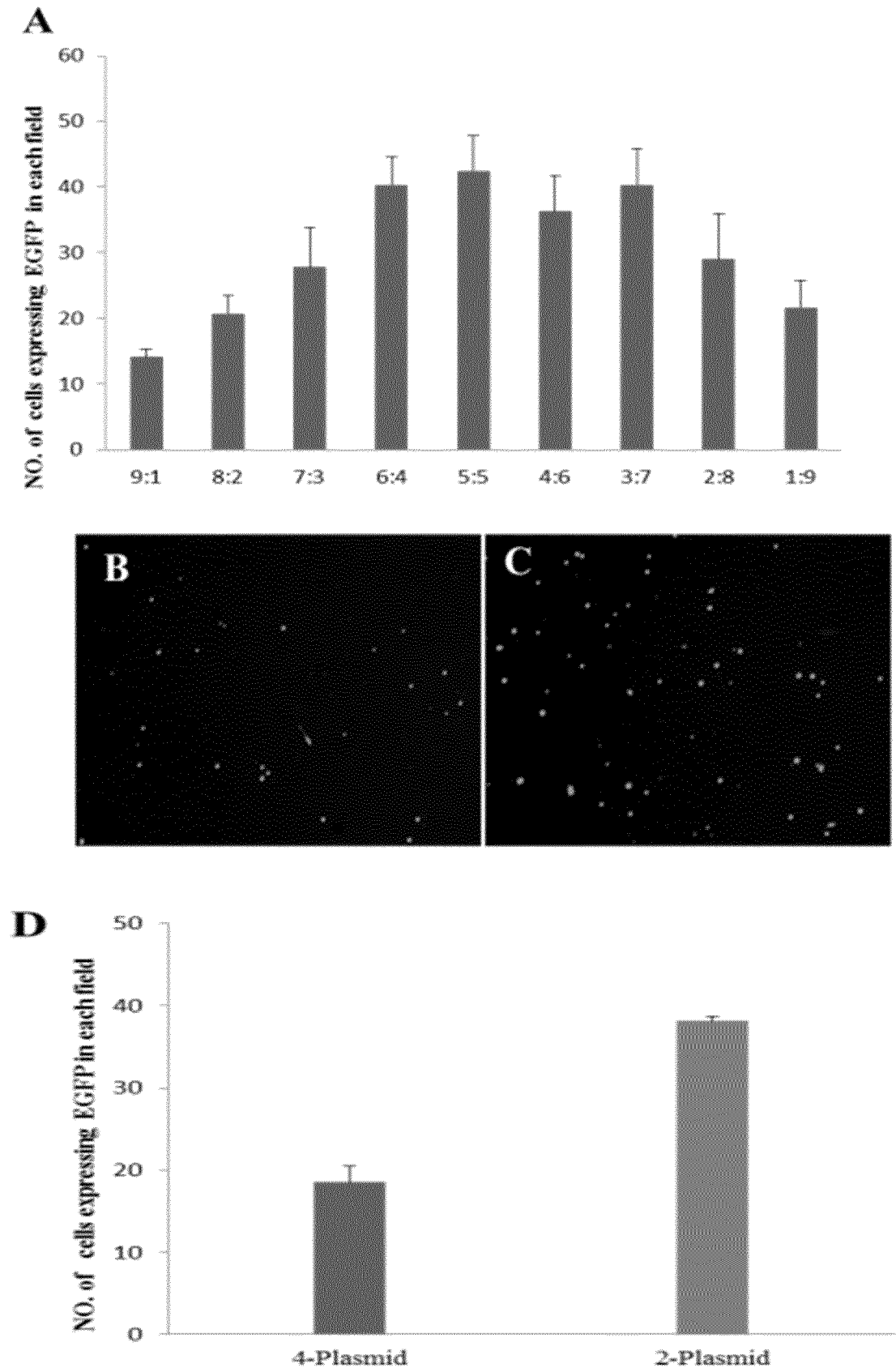


FIGURE 5

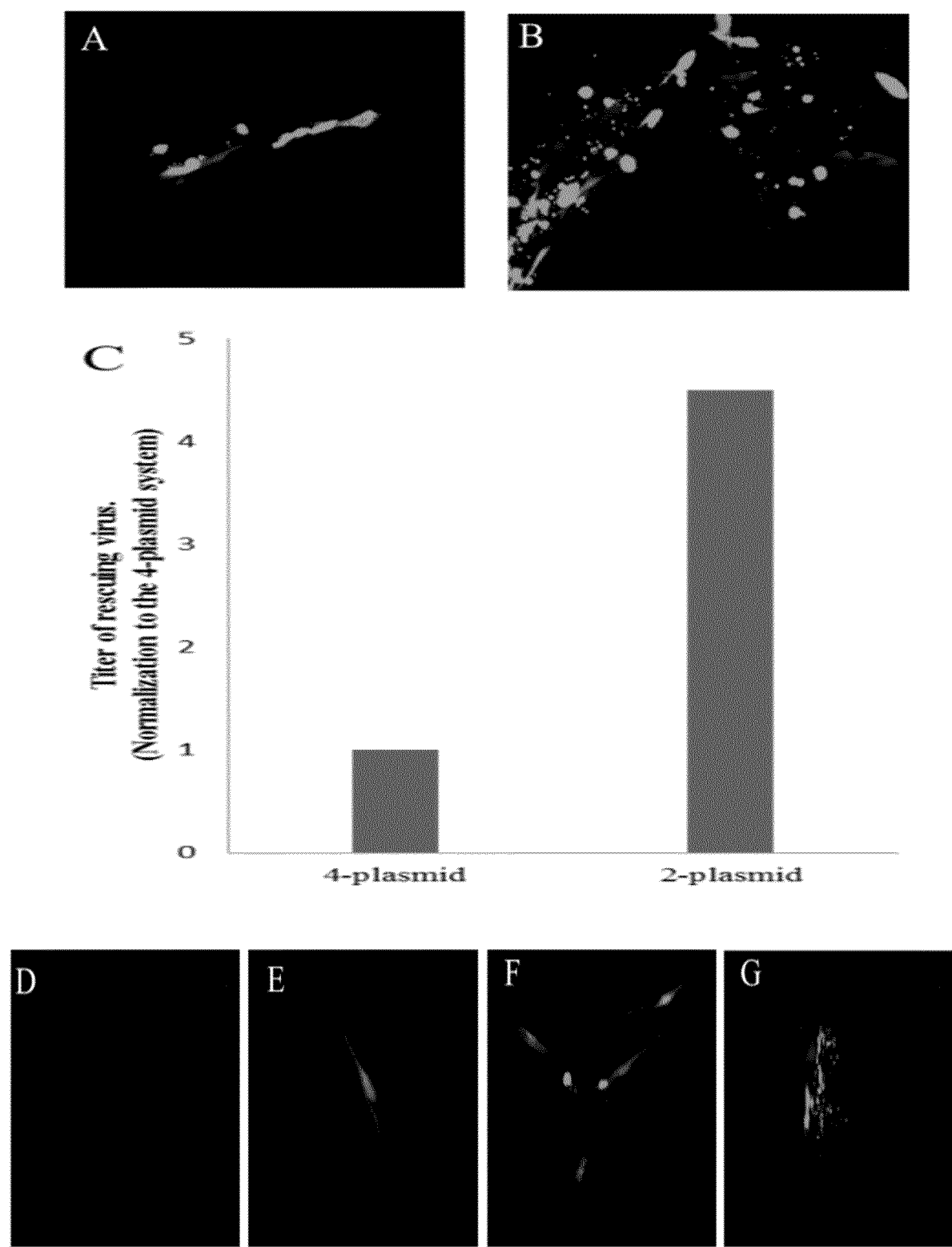


FIGURE 6

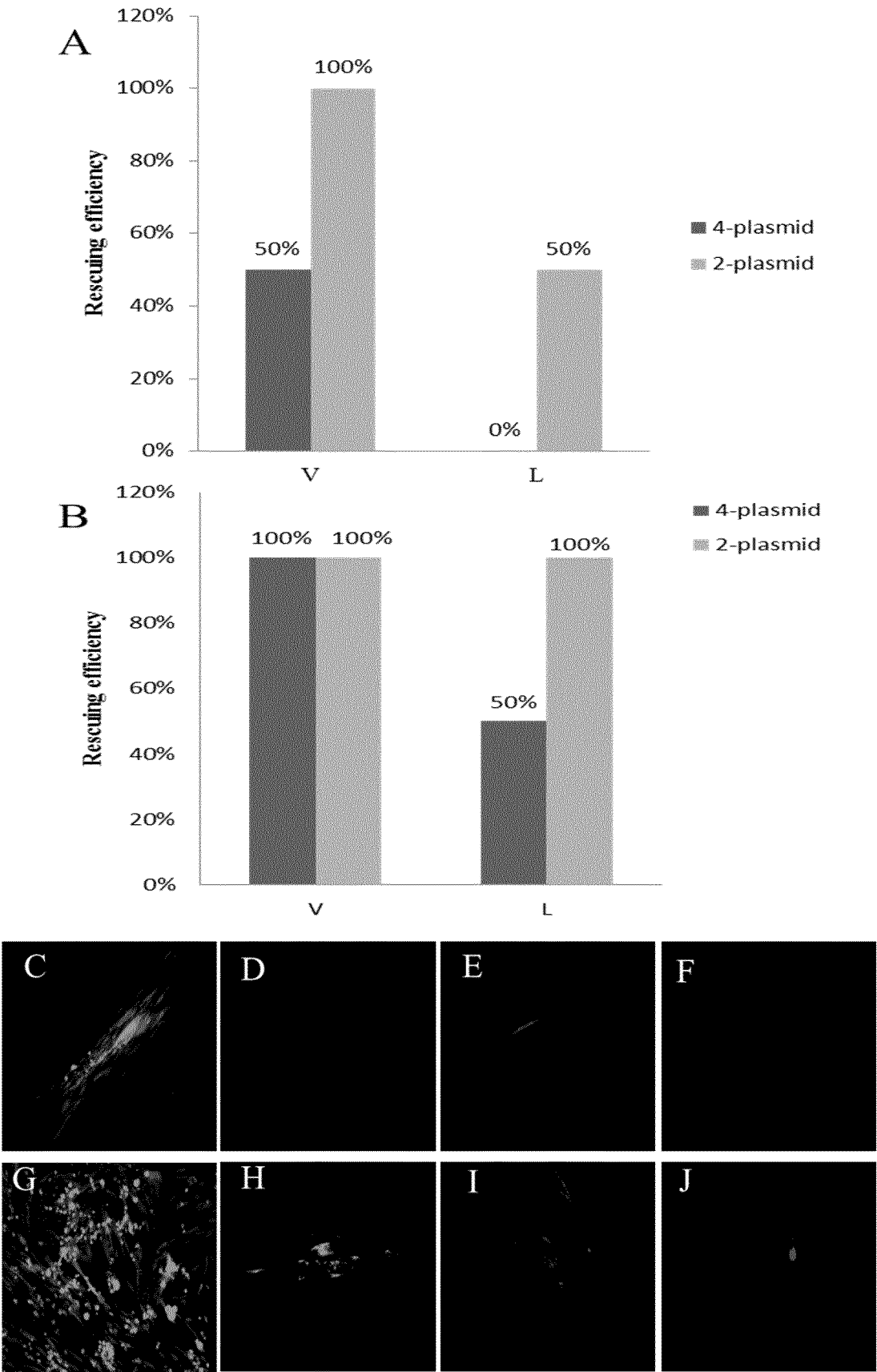


FIGURE 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/054903

A. CLASSIFICATION OF SUBJECT MATTER INV. C12N7/02 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data, BIOSIS, Sequence Search		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/046216 A2 (JOSHI VISHWAS [IN]) 4 April 2013 (2013-04-04) abstract; figure 4; tables 1-4 page 5 - page 13; figures 1-3, 5, 6; sequence HQ197753.1 <div style="text-align: center; margin-top: 10px;"> ----- -/-- </div>	1-15
<div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex. </div>		
* Special categories of cited documents :		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search	Date of mailing of the international search report	
18 April 2018	30/04/2018	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Schulz, Regine	

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/054903

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JIAXU LIU ET AL: "The deletion of an extra six nucleotides in the 5'-untranslated region of the nucleoprotein gene of Newcastle disease virus NA-1 decreases virulence", BMC VETERINARY RESEARCH, BIOMED CENTRAL, LONDON, GB, vol. 10, no. 1, 21 December 2014 (2014-12-21), page 964, XP021209897, ISSN: 1746-6148, DOI: 10.1186/S12917-014-0305-5	11,12
A	abstract; figures 1-4 page 2 - page 6	1-10, 13-15
X	----- WITKO S E ET AL: "An efficient helper-virus-free method for rescue of recombinant paramyxoviruses and rhadoviruses from a cell line suitable for vaccine development", JOURNAL OF VIROLOGICAL METHODS, ELSEVIER BV, NL, vol. 135, no. 1, 1 July 2006 (2006-07-01), pages 91-101, XP027892404, ISSN: 0166-0934 [retrieved on 2006-07-01] abstract; figures 1-3; tables 1-3 page 91	11,12
A	----- WANG JIANZHONG ET AL: "Development of a reverse genetics system based on RNA polymerase II for Newcastle disease virus genotype VII", VIRUS GENES, KLUWER ACADEMIC PUBLISHERS, BOSTON, US, vol. 50, no. 1, 11 November 2014 (2014-11-11), pages 152-155, XP035462346, ISSN: 0920-8569, DOI: 10.1007/S11262-014-1137-X [retrieved on 2014-11-11] cited in the application abstract; figure 1; table 1 page 153 - page 154	1-15
A	----- US 2014/287496 A1 (WEN GUOYUAN [CN] ET AL) 25 September 2014 (2014-09-25) abstract; figures 1, 6 page 1, paragraph [0005] - page 3, paragraph [0040] ----- -/--	1-15

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/054903

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YANLONG JIANG ET AL: "Plasmids driven minigenome rescue system for Newcastle disease virus V4 strain", MOLECULAR BIOLOGY REPORTS ; AN INTERNATIONAL JOURNAL ON MOLECULAR AND CELLULAR BIOLOGY, KLUWER ACADEMIC PUBLISHERS, DO, vol. 36, no. 7, 15 November 2008 (2008-11-15), pages 1909-1914, XP019732348, ISSN: 1573-4978 abstract; figures 1-4 page 1910 - page 1913 -----	1-15
T	NEUMANN G ET AL: "An improved reverse genetics system for influenza A virus generation and its implications for vaccine production", PROCEEDINGS NATIONAL ACADEMY OF SCIENCES PNAS, NATIONAL ACADEMY OF SCIENCES, US, vol. 102, no. 46, 1 November 2005 (2005-11-01), pages 16825-16829, XP002428349, ISSN: 0027-8424, DOI: 10.1073/PNAS.0505587102 abstract; figure 1 page 16826 -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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