Vaccine
Manuscript Draft

Manuscript Number: JVAC-D-18-00728

Title: Generation of a recombinant Newcastle disease virus expressing two foreign genes for use as a multivalent vaccine and gene therapy vector

Article Type: Short Communication

Section/Category: Vaccine Technology (Vectors/Adjuvants/Delivery Systems/Nanotechnology)

Keywords: NDV; RFP; GFP; multivalent vector; independent transcription unit; internal ribosomal entry site

Abstract: Newcastle disease virus (NDV) has been used as a vector in the development of vaccines and gene therapy. A majority of these NDV vectors express only a single foreign gene through either an independent transcription unit (ITU) or an internal ribosomal entry site (IRES). In the present study, we combined the ITU and IRES methods to generate a novel NDV LaSota strain-based recombinant virus vectoring the red fluorescence protein (RFP) and the green fluorescence protein (GFP) genes. Biological assessments of the recombinant virus, rLS/IRES-RFP/GFP, showed that it was slightly attenuated in vivo, yet maintained similar growth dynamics and viral yields in vitro when compared to the parental LaSota virus. Expression of both the RFP and GFP was detected from the rLS/IRES-RFP/GFP virus-infected DF-1 cells by fluorescence microscopy. These data suggest that the rLS/IRES-RFP/GFP virus may be used as a multivalent vector for the development of vaccines and gene therapy agents.

ABSTRACT

Newcastle disease virus (NDV) has been used as a vector in the development of vaccines and gene therapy. A majority of these NDV vectors express only a single foreign gene through either an independent transcription unit (ITU) or an internal ribosomal entry site (IRES). In the present study, we combined the ITU and IRES methods to generate a novel NDV LaSota strain-based recombinant virus vectoring the red fluorescence protein (RFP) and the green fluorescence protein (GFP) genes. Biological assessments of the recombinant virus, rLS/IRES-RFP/GFP, showed that it was slightly attenuated in *vivo*, yet maintained similar growth dynamics and viral yields *in vitro* when compared to the parental LaSota virus. Expression of both the RFP and GFP was detected from the rLS/IRES-RFP/GFP virus-infected DF-1 cells by fluorescence microscopy. These data suggest that the rLS/IRES-RFP/GFP virus may be used as a multivalent vector for the development of vaccines and gene therapy agents.

*Title Page

Generation of a recombinant Newcastle disease virus expressing two foreign genes for use as a multivalent vaccine and gene therapy vector

Haixia Hu[1,2], Jason P. Roth[2,a], and Qingzhong Yu[2]*

[1] College of Animal Science and Technology, Southwest University, Chongqing 400715, China

[2] Southeast Poultry Research Laboratory, US National Poultry Research Center, Agricultural Research Services, United States Department of Agriculture, 934 College Station Road, Athens, GA 30605, USA

* Corresponding author. US National Poultry Research Center, Agricultural Research Services, United States Department of Agriculture, Athens, 30605, USA

Tel: +1 706 546 3628; Fax: +1 706 546 3161.

E-mail address: qingzhong.yu@ars.usda.gov

a. Current address: Boehringer-Ingelheim, 1730 Olympic Dr., Athens, GA 30601, USA

*Manuscript
Click here to view linked References

1 **Generation of a recombinant Newcastle disease virus expressing two foreign**
2 **genes for use as a multivalent vaccine and gene therapy vector**
3
4
5
6
7 Haixia Hu[1,2], Jason P. Roth[2,a], and Qingzhong Yu[2]*
8
9
10
11 [1] College of Animal Science and Technology, Southwest University, Chongqing 400715, China
12
13 [2] Southeast Poultry Research Laboratory, US National Poultry Research Center, Agricultural
14 Research Services, United States Department of Agriculture, 934 College Station Road, Athens,
15 GA 30605, USA
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30 * Corresponding author. US National Poultry Research Center, Agricultural Research Services,

31 United States Department of Agriculture, Athens, 30605, USA

32 Tel: +1 706 546 3628; Fax: +1 706 546 3161.

33 E-mail address: qingzhong.yu@ars.usda.gov
34
35
36
37 a. Current address: Boehringer-Ingelheim, 1730 Olympic Dr., Athens, GA 30601, USA
38
39
40
41
42
43

ABSTRACT

Newcastle disease virus (NDV) has been used as a vector in the development of vaccines and gene therapy. A majority of these NDV vectors express only a single foreign gene through either an independent transcription unit (ITU) or an internal ribosomal entry site (IRES). In the present study, we combined the ITU and IRES methods to generate a novel NDV LaSota strain-based recombinant virus vectoring the red fluorescence protein (RFP) and the green fluorescence protein (GFP) genes. Biological assessments of the recombinant virus, rLS/IRES-RFP/GFP, showed that it was slightly attenuated in *vivo*, yet maintained similar growth dynamics and viral yields *in vitro* when compared to the parental LaSota virus. Expression of both the RFP and GFP was detected from the rLS/IRES-RFP/GFP virus-infected DF-1 cells by fluorescence microscopy. These data suggest that the rLS/IRES-RFP/GFP virus may be used as a multivalent vector for the development of vaccines and gene therapy agents.

KEYWORDS: NDV; RFP; GFP; multivalent vector; independent transcription unit; internal ribosomal entry site.

Newcastle disease virus (NDV) is an enveloped, non-segmented, negative-stranded RNA virus and has been classified as avian paramyxovirus serotype 1 (APMV-1) in the genus *Avulavirus* of the family *Paramyxoviridae* [1]. NDV is an avian pathogen that can cause local infections in humans, however, there is no current data to suggest that NDV can be transmitted human-to-human [2, 3]. The NDV genome consists of approximately 15.2 kb and contains six transcriptional units, encoding the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large polymerase protein (L), in that order [4]. The genomic RNA, together with the NP, P, and L proteins, form the ribonucleoprotein complex (RNP), which serves as the active template for transcription and replication of the viral genome [5, 6].

Since reverse genetics technology was first used to rescue infectious NDV from recombinant cDNA in 1999 [7, 8], many NDV clones have been developed and used as vectors to express foreign genes for vaccine or gene therapy purposes [9-15]. Most of these NDV vectors express only a single foreign gene from an additional independent transcription unit (ITU) that is inserted between other native viral transcription units in the NDV genome [15]. Recently we

77 developed a novel approach to express a foreign gene from within native viral transcription units
78 using an internal ribosomal entry site (IRES). In this case the red fluorescence protein (RFP)
79 gene was expressed from a second open reading frame (ORF) located in all six native
80 transcriptional units, separately, using an IRES [16]. The addition of the second ORF did not
81 significantly affect viral replication, and the level of foreign gene expression could be regulated
82 by inserting the second ORF into different native viral transcription units.
83
84 In this study, we combined the ITU and IRES approaches to develop a multi-foreign gene
85 expression vector. The NDV LaSota (LS) strain-based infectious clone, pLS/aMPV-C G [17],
86 was used as a backbone to construct a recombinant cDNA clone containing both RFP and GFP
87 genes (Figure 1). First, the ORF of the aMPV-C G gene in the pLS/aMPV-C G clone was
88 replaced with the GFP ORF, amplified from the pAAV-hrGFP plasmid (Agilent Technologies,
89 Santa Clara, CA), using an In-Fusion® PCR cloning kit (Clontech, Mountain View, CA), which
90 resulted in the pLS/GFP subclone. Second, the RFP gene, amplified from the pCMV-Ds-Red-
91 Express plasmid (Clontech), was cloned downstream of the IRES sequence in pIRES-hrGFP-2a
92 vector (Clontech). Finally, the IRES and the RFP ORF sequences were amplified and cloned
93 downstream of the NDV F ORF in the pLS/GFP vector as a 2nd ORF in the NDV F transcription
94 unit using the In-Fusion® PCR Cloning Kit (Clontech). The resulting recombinant clone,
95 designated as pLS/IRES-RFP/GFP, was amplified in Stbl2 cells at 30°C for 24 hours and
96 purified using a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA).
97
98 After co-transfection of the pLS/IRES-RFP/GFP clone and the supporting plasmids,
99 encoding for the NDV NP, P, and L proteins, into HEp-2 cells, the rescued the LaSota strain-
100 based recombinant virus vectoring the RFP and GFP genes, designated as rLS/IRES-RFP/GFP,
101 was subsequently amplified in SPF chicken embryonated eggs as described previously [18]. The
102 rLS/IRES-RFP/GFP virus was purified and further propagated in SPF chicken embryonated
103 eggs. The fidelity of the rescued rLS/IRES-RFP/GFP virus was confirmed by sequencing the
104 isolated viral genome (data not shown).
105
106 To determine if the addition of the two foreign genes, RFP and GFP, affects the viral
107 replication and pathogenicity of the rLS/IRES-RFP/GFP virus, the hemagglutination (HA), 50%

108 tissue culture infectious dose ($TCID_{50}$), 50% egg infective dose (EID_{50}), mean death time
109 (MDT), and intracerebral pathogenicity index (ICPI) were examined [19]. As shown in Table 1,
110 the recombinant virus appears to be slightly attenuated with a lower ICPI (0.0) in day-old
111 chickens, longer MDT (>150hs) in embryos, and greater than a half-log reduction in viral yield
112 in DF-1 cells compared to the parental LaSota strain. The viral yields of the recombinant virus
113 grown in embryonated eggs, measured by EID_{50} and HA, were comparable to the yields of the
114 parental LaSota strain (Table 1). Overall, the replication rate of rLS/IRES-RFP/GFP appears to
115 be similar when compared to the replication rate of LaSota in DF-1 cells (Fig. 2).
116

117 The co-expression of the RFP and GFP proteins from rLS/ IRES-RFP/GFP infected DF-1
118 cells at 24 h post-infection was examined by fluorescence microscopy at 100 x magnification
119 (Nikon, Eclipse Ti, Melville, NY) [17]. As shown in Fig. 3, both GFP (Fig. 3b) and RFP (Fig.
120 3c) expression was observed. After merging both fluorescent images (Fig.3d), GFP and RFP co-
121 localized to the same infected cells as seen by NDV induced viral cytopathic effects (CPE)
122 observed under bright field (Fig. 3a). It is notable that there are a few infected cells that express
123 GFP but not RFP that most likely can be attributed to the different promoters that drive the
124 expression of the reporter genes. The GFP gene is inserted into the NDV genome as its own ITU
125 and therefore should be expressed readily. However, the RFP gene is inserted as a second ORF
126 downstream of an IRES inside a native transcription unit. The expression of a foreign gene from
127 an IRES appears to be slightly inconsistent as several cells expressing GFP but not RFP can be
128 seen.
129

130 Several NDV based recombinant viruses expressing a foreign gene have been devloped and
131 evaluated as vaccine candidates in clinical trials resulting in varying levels of the host's immune
132 response and protection against the targeted pathogen [17, 20-31]. Although the host's response
133 to vaccination and, ultimately, a protective immunity is influenced by many factors, antigenicity
134 and expression efficiency of the foreign antigen are undoubtedly the most important. In most
135 reported NDV vaccine candidates, the recombinant vectors only express one foreign gene
136 through an independent transcription unit. The insufficient immune response and lack of
137 protection against a targeted pathogen conferred by these vectored vaccine candidates suggest
138 that a single antigen from the pathogen or its expression level from the vector may not be

139 adequate. Alternatively, two or more antigenic components from a pathogen may be required to
140 achieve an improved or complete protection against challenge of said pathogen [32-34]. In this
141 study, we have shown that it is possible to express two foreign genes simultaneously from a
142 single vector that could be used to deliver the necessary antigenic components from one
143 pathogen to induce a stronger immune response and improve the protective efficacy.

144 Studies that express two foreign genes from a single NDV vector to be used as experimental
145 vaccine candidates have been developed resulting in observed improvements in protective
146 efficacy against pathogenic challenge [33-35]. However, these foreign antigens were expressed
147 through two additional ITUs in the NDV genome. Previous studies have demonstrated that the
148 addition of an ITU in the viral genome downregulates viral gene transcription downstream of the
149 inserted ITU that may subsequently attenuate viral replication and pathogenicity and, ultimately,
150 decrease foreign gene expression [36-40]. Whereas, the NDV vector developed in this study
151 expresses one foreign gene through an additional ITU and the other through an IRES as a second
152 ORF within a native transcription unit, which usually does not notably affect viral replication
153 efficiency [16]. More importantly, expression of the second foreign gene, downstream of an
154 IRES, could be regulated by selecting an insertion site in the NDV genome relative to the desired
155 expression level to meet the needs of multivalent vaccines and anticancer therapy [16].

156 Multivalent vaccination is the preferred practice in the poultry industry to protect the birds
157 from common infectious diseases with little handling as possible. One of the advantages in
158 administering multivalent vaccines is the reduction in vaccination costs, especially for large
159 producers. However, a general concern is that the combination of live-recombinant vaccines
160 with live-attenuated viruses may interfere with the other's replication in the host and compromise
161 protective efficacy [41, 42]. The NDV vector developed in this study containing two foreign
162 genes could express viral antigens from two different pathogens and be used as a trivalent
163 vaccine against NDV and the other two targeted diseases. In this case, this trivalent vaccine is
164 one isolated replicating virus population with regulated foreign gene expression that should not
165 interfere with itself nor induction of the host's immune response [43].

166 Several NDV based recombinant vectors expressing cytokines or anticancer factors have
167 been evaluated as anticancer agents in tumor models [44-49]. Again, most of these NDV
168 recombinant vectors express only a single cytokine or anticancer factor and varying levels of

169 therapeutic effects were achieved in clinical trials. It would be interesting if a combination of two
170 or more cytokines or anticancer factors expressed from a single NDV vector, through the ITU
171 and IRES approaches, improved the efficacy of potential anticancer therapies.

172 In summary, we developed a novel NDV vector co-expressing the GFP and RFP
173 reporters through a combination of the ITU and IRES approaches in this study. The recombinant
174 NDV vectoring the two foreign genes was slightly attenuated when compared to its parental
175 virus. There was no discernible interference with viral replication rates or expression of the two
176 foreign genes. Our results suggest that the NDV LaSota strain-based two-foreign gene
177 expression vector is reliable and may be used in the development of multivalent vaccines against
178 veterinary viral diseases, and may also have potential applications in human and veterinary gene
179 therapy or anticancer treatments.

180 ACKNOWLEDGEMENTS
181
182 The authors wish to thank Xiuqin Xia and Fenglan Li for excellent technical assistance and Ron
183 Iorio for a gift of anti-NDV HN monoclonal antibody. This research was supported by USDA,
184 ARS project 6612-32000-067-00D, and National Natural Science Foundation of China (C0809-
185 31400784).
186 Conflict of interest: The authors declare that they have no conflicts of interest.

187
188
189
190 REFERENCE
191

192 [1] Amarasinghe GK, Bao Y, Basler CF, Bavari S, Beer M, Bejerman N, et al. Taxonomy of the
193 order Mononegavirales: update 2017. Arch Virol. 2017.
194 [2] Miller PJ, Koch G. Newcastle Disease. In: Swayne DE, Glisson JR, McDougald LR, Nolan
195 LK, Suarez DL, Nair V, editors. Diseases of Poultry. 13th ed. Ames, Iowa, USA: Wiley-
196 Blackwell Publishing; 2013. p. 98-107.
197 [3] Swayne DE, King DJ. Avian influenza and Newcastle disease. J Am Vet Med Assoc.
198 2003;222:1534-40.
199 [4] Samal SK. Newcastle disease and ralated avian paramyxoviruses. In: Samal SK, editor. The
200 Biology of Paramyxoviruses. Norfolk, UK: Caister Academic press; 2011. p. 69-114.
201 [5] Lamb RA, Parks GD. Paramyxoviridae. In: Knipe DM, Howley PM, Cohen JI, Griffin DE,
202 Lamb RA, Marti MA, et al., editors. Fields Virology. Sixth ed. Philadelphia, PA, USA:
203 Lippincott Williams & Wilkins; 2013. p. 957-95.
204 [6] Peeters BP, Gruijthuijsen YK, de Leeuw OS, Gielkens AL. Genome replication of Newcastle
205 disease virus: involvement of the rule-of-six. Arch Virol. 2000;145:1829-45.

206 [7] Peeters BP, de Leeuw OS, Koch G, Gielkens AL. Rescue of Newcastle disease virus from
207 cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for
208 virulence. J Virol. 1999;73:5001-9.
209 [8] Romer-Oberdorfer A, Mundt E, Mebatsion T, Buchholz UJ, Mettenleiter TC. Generation of
210 recombinant lentogenic Newcastle disease virus from cDNA. J Gen Virol. 1999;80 (Pt
211 11):2987-95.
212 [9] Bukreyev A, Collins PL. Newcastle disease virus as a vaccine vector for humans. Current
213 opinion in molecular therapeutics. 2008;10:46-55.
214 [10] Huang Z, Elankumaran S, Panda A, Samal SK. Recombinant Newcastle disease virus as a
215 vaccine vector. Poultry Science. 2003;82:899-906.
216 [11] Kim SH, Samal SK. Newcastle Disease Virus as a Vaccine Vector for Development of
217 Human and Veterinary Vaccines. Viruses. 2016;8.
218 [12] Kim SH, Samal SK. Reverse Genetics for Newcastle Disease Virus as a Vaccine Vector.
219 Curr Protoc Microbiol. 2018;48:18 5 1- 5 2.
220 [13] Schirrmacher V, Fournier P. Newcastle disease virus: a promising vector for viral therapy,
221 immune therapy, and gene therapy of cancer. Methods Mol Biol. 2009;542:565-605.
222 [14] Vigil A, Martinez O, Chua MA, Garcia-Sastre A. Recombinant Newcastle disease virus as a
223 vaccine vector for cancer therapy. Molecular therapy : the journal of the American Society of
224 Gene Therapy. 2008;16:1883-90.
225 [15] Yu Q. Have we found an optimal insertion site in a Newcastle disease virus vector to
226 express a foreign gene for vaccine and gene therapy purposes? British Journal of Virology.
227 2015;2:15-8.
228 [16] Zhang Z, Zhao W, Li D, Yang J, Zsak L, Yu Q. Development of a Newcastle disease virus
229 vector expressing a foreign gene through an internal ribosomal entry site provides direct proof
230 for a sequential transcription mechanism. J Gen Virol. 2015;96:2028-35.
231 [17] Hu H, Roth JP, Estevez CN, Zsak L, Liu B, Yu Q. Generation and evaluation of a
232 recombinant Newcastle disease virus expressing the glycoprotein (G) of avian metapneumovirus
233 subgroup C as a bivalent vaccine in turkeys. Vaccine. 2011;29:8624-33.
234 [18] Estevez CN, King DJ, Seal B, Yu Q. Evaluation of Newcastle disease virus chimeras
235 expressing the Hemagglutinin-Neuraminidase protein of velogenic strains in the context of a
236 mesogenic recombinant virus backbone. Virus Res. 2007;129:182-90.
237 [19] Alexander DJ. Newcastle Disease Virus and other Avian Paramyxoviruses. In: Swayne D,
238 Glisson JR, Jackwood MW, Pearson JE, Reed WM, editors. A laboratory manual for the
239 isolation and identification of avian pathogens. 4th Edition ed. Kennett Square, PA: American
240 Association of Avian Pathologists; 1998. p. 156-63.
241 [20] DiNapoli JM, Nayak B, Yang L, Finneyfrock BW, Cook A, Andersen H, et al. Newcastle
242 disease virus-vectored vaccines expressing the hemagglutinin or neuraminidase protein of H5N1
243 highly pathogenic avian influenza virus protect against virus challenge in monkeys. J Virol.
244 2010;84:1489-503.
245 [21] Eldemery F, Li Y, Yu Q, van Santen VL, Toro H. Infectious Bronchitis Virus S2 of 4/91
246 Expressed from Recombinant Virus Does Not Protect Against Ark-Type Challenge. Avian Dis.
247 2017;61:397-401.
248 [22] Ge J, Wang X, Tian M, Gao Y, Wen Z, Yu G, et al. Recombinant Newcastle disease viral
249 vector expressing hemagglutinin or fusion of canine distemper virus is safe and immunogenic in
250 minks. Vaccine. 2015;33:2457-62.

251 [23] Huang Z, Elankumaran S, Yunus AS, Samal SK. A recombinant Newcastle disease virus
252 (NDV) expressing VP2 protein of infectious bursal disease virus (IBDV) protects against NDV
253 and IBDV. J Virol. 2004;78:10054-63.
254 [24] Kanabagatte Basavarajappa M, Kumar S, Khattar SK, Gebreluul GT, Paldurai A, Samal SK.
255 A recombinant Newcastle disease virus (NDV) expressing infectious laryngotracheitis virus
256 (ILTV) surface glycoprotein D protects against highly virulent ILTV and NDV challenges in
257 chickens. Vaccine. 2014;32:3555-63.
258 [25] Kim SH, Chen S, Jiang X, Green KY, Samal SK. Newcastle disease virus vector producing
259 human norovirus-like particles induces serum, cellular, and mucosal immune responses in mice.
260 J Virol. 2014;88:9718-27.
261 [26] Kong D, Wen Z, Su H, Ge J, Chen W, Wang X, et al. Newcastle disease virus-vectored
262 Nipah encephalitis vaccines induce B and T cell responses in mice and long-lasting neutralizing
263 antibodies in pigs. Virology. 2012;432:327-35.
264 [27] Romer-Oberdorfer A, Veits J, Helferich D, Mettenleiter TC. Level of protection of chickens
265 against highly pathogenic H5 avian influenza virus with Newcastle disease virus based live
266 attenuated vector vaccine depends on homology of H5 sequence between vaccine and challenge
267 virus. Vaccine. 2008;26:2307-13.
268 [28] Schroer D, Veits J, Keil G, Romer-Oberdorfer A, Weber S, Mettenleiter TC. Efficacy of
269 Newcastle disease virus recombinant expressing avian influenza virus H6 hemagglutinin against
270 Newcastle disease and low pathogenic avian influenza in chickens and turkeys. Avian Dis.
271 2011;55:201-11.
272 [29] Toro H, Zhao W, Breedlove C, Zhang Z, Yub Q. Infectious bronchitis virus S2 expressed
273 from recombinant virus confers broad protection against challenge. Avian Dis. 2014;58:83-9.
274 [30] Yu Q, Spatz S, Li Y, Yang J, Zhao W, Zhang Z, et al. Newcastle disease virus vectored
275 infectious laryngotracheitis vaccines protect commercial broiler chickens in the presence of
276 maternally derived antibodies. Vaccine. 2017;35:789-95.
277 [31] Zhao W, Spatz S, Zhang Z, Wen G, Garcia M, Zsak L, et al. Newcastle disease virus (NDV)
278 recombinants expressing infectious laryngotracheitis virus (ILTV) glycoproteins gB and gD
279 protect chickens against ILTV and NDV challenges. J Virol. 2014;88:8397-406.
280 [32] Hu H, Roth JP, Zsak L, Yu Q. Engineered Newcastle disease virus expressing the F and G
281 proteins of AMPV-C confers protection against challenges in turkeys. Sci Rep. 2017;7:4025.
282 [33] Khattar SK, Manoharan V, Bhattarai B, LaBranche CC, Montefiori DC, Samal SK. Mucosal
283 Immunization with Newcastle Disease Virus Vector Coexpressing HIV-1 Env and Gag Proteins
284 Elicits Potent Serum, Mucosal, and Cellular Immune Responses That Protect against Vaccinia
285 Virus Env and Gag Challenges. MBio. 2015;6:e01005.
286 [34] Ramp K, Veits J, Deckers D, Rudolf M, Grund C, Mettenleiter TC, et al. Coexpression of
287 avian influenza virus H5 and N1 by recombinant Newcastle disease virus and the impact on
288 immune response in chickens. Avian Dis. 2011;55:413-21.
289 [35] Puhler F, Willuda J, Puhlmann J, Mumberg D, Romer-Oberdorfer A, Beier R. Generation of
290 a recombinant oncolytic Newcastle disease virus and expression of a full IgG antibody from two
291 transgenes. Gene Ther. 2008;15:371-83.
292 [36] Krishnamurthy S, Huang Z, Samal SK. Recovery of a virulent strain of newcastle disease
293 virus from cloned cDNA: expression of a foreign gene results in growth retardation and
294 attenuation. Virology. 2000;278:168-82.

295 [37] Skiadopoulos MH, Surman SR, Durbin AP, Collins PL, Murphy BR. Long nucleotide
296 insertions between the HN and L protein coding regions of human parainfluenza virus type 3
297 yield viruses with temperature-sensitive and attenuation phenotypes. Virology. 2000;272:225-34.
298 [38] Wertz GW, Moudy R, Ball LA. Adding genes to the RNA genome of vesicular stomatitis
299 virus: positional effects on stability of expression. J Virol. 2002;76:7642-50.
300 [39] Zhao H, Peeters BP. Recombinant Newcastle disease virus as a viral vector: effect of
301 genomic location of foreign gene on gene expression and virus replication. J Gen Virol.
302 2003;84:781-8.
303 [40] Zhao W, Zhang Z, Zsak L, Yu Q. P and M gene junction is the optimal insertion site in
304 Newcastle disease virus vaccine vector for foreign gene expression. J Gen Virol. 2015;96:40-5.
305 [41] Gelb J, Jr., Ladman BS, Licata MJ, Shapiro MH, Campion LR. Evaluating viral interference
306 between infectious bronchitis virus and Newcastle disease virus vaccine strains using
307 quantitative reverse transcription-polymerase chain reaction. Avian Dis. 2007;51:924-34.
308 [42] Spier RE. Multivalent vaccines: prospects and challenges. Folia Microbiol (Praha).
309 1997;42:105-12.
310 [43] Vidor E. The nature and consequences of intra- and inter-vaccine interference. J Comp
311 Pathol. 2007;137 Suppl 1:S62-6.
312 [44] Bai FL, Yu YH, Tian H, Ren GP, Wang H, Zhou B, et al. Genetically engineered Newcastle
313 disease virus expressing interleukin-2 and TNF-related apoptosis-inducing ligand for cancer
314 therapy. Cancer biology & therapy. 2014;15:1226-38.
315 [45] Bu X, Li M, Zhao Y, Liu S, Wang M, Ge J, et al. Genetically engineered Newcastle disease
316 virus expressing human interferon-lambda1 induces apoptosis in gastric adenocarcinoma cells
317 and modulates the Th1/Th2 immune response. Oncol Rep. 2016;36:1393-402.
318 [46] Niu Z, Bai F, Sun T, Tian H, Yu D, Yin J, et al. Recombinant Newcastle Disease virus
319 Expressing IL15 Demonstrates Promising Antitumor Efficiency in Melanoma Model.
320 Technology in cancer research & treatment. 2014.
321 [47] Vigil A, Park MS, Martinez O, Chua MA, Xiao S, Cros JF, et al. Use of reverse genetics to
322 enhance the oncolytic properties of Newcastle disease virus. Cancer Res. 2007;67:8285-92.
323 [48] Wu Y, He J, An Y, Wang X, Liu Y, Yan S, et al. Recombinant Newcastle disease virus
324 (NDV/Anh-IL-2) expressing human IL-2 as a potential candidate for suppresses growth of
325 hepatoma therapy. J Pharmacol Sci. 2016;132:24-30.
326 [49] Wu Y, He J, Geng J, An Y, Ye X, Yan S, et al. Recombinant Newcastle disease virus
327 expressing human TRAIL as a potential candidate for hepatoma therapy. Eur J Pharmacol.
328 2017;802:85-92.
329
330

Tables

Table 1. Biological assessments of the rLS/IRES-RFP/GFP and parental LaSota viruses

Virus	MDT[a]	ICPI[b]	HA[c]	EID_{50}[d]	$TCID_{50}$[e]
LaSota	110hs	0.15	1024	2.37×10^{9}	9.88×10^{8}
rLS/IRES-RFP/GFP	>150hs	0	512	2.37×10^{9}	1.76×10^{8}

[a] MDT: Mean death time assay in embryonated chicken eggs.
[b] ICPI: Intracerebral pathogenicity index assay in day-old chickens.
[c] HA: Hemagglutination assay.
[d] EID_{50}: 50% egg infective dose assay in embryonated chicken eggs.
[e] $TCID_{50}$: 50% tissue infectious dose assay in DF-1 cells.

Figures

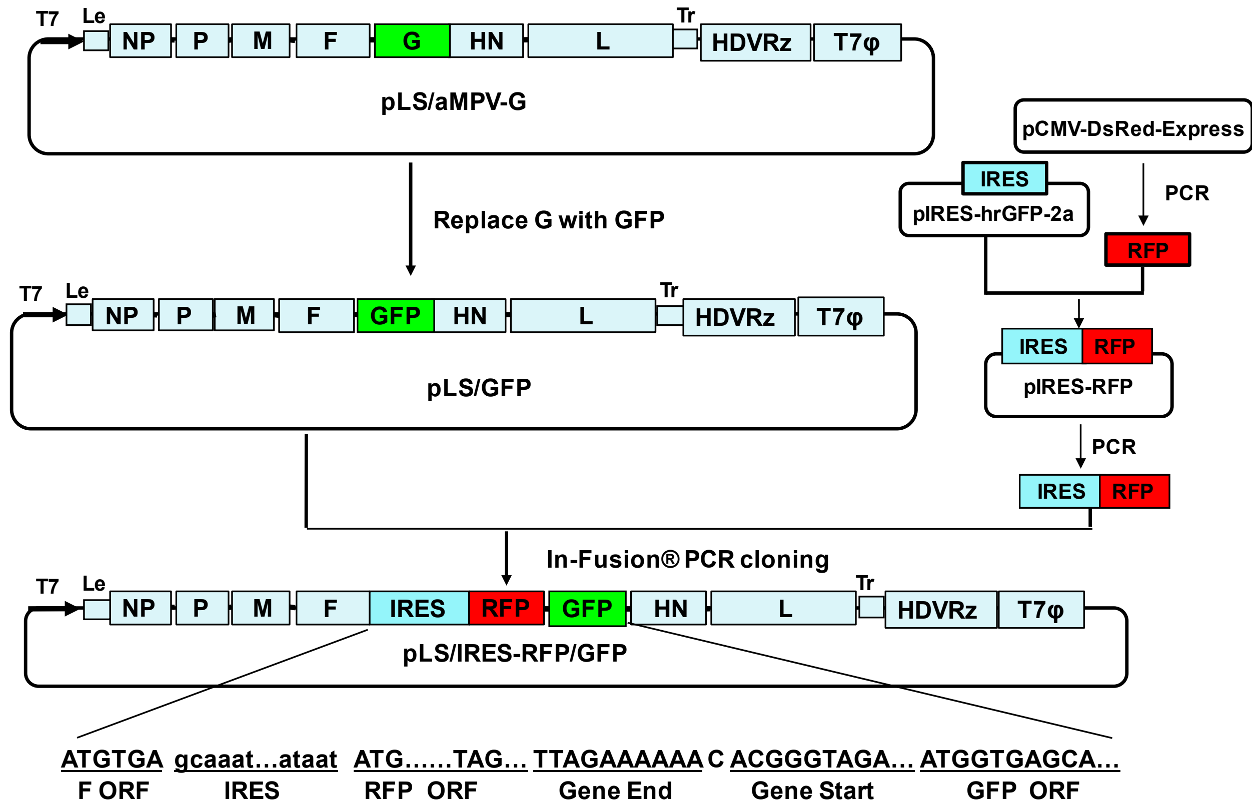

Fig. 1. Schematic representation of pLS/IRES-RFP/GFP construction. The ORF of the aMPV-C G gene in the pLS/aMPV-C G clone was replaced with the GFP ORF, amplified from the pAAV-hrGFP plasmid (Agilent Technologies, Santa Clara, CA), using an In-Fusion® PCR cloning kit (Clontech, Mountain View, CA), resulting in a subclone pLS/GFP. The RFP ORF, amplified from the plasmid pCMV-Ds-Red-Express (Clontech), was cloned downstream of the IRES sequence in the pIRES-hrGFP-2a vector (Clontech). Subsequently, the IRES and the RFP ORF sequences were amplified and cloned downstream of the NDV F ORF in the pLS/GFP vector using an In-Fusion® PCR Cloning Kit (Clontech). The NDV Gene End and Gene Start signal sequences and the RFP and GFP sequences are underlined. The direction of the T7 promoter is indicated by a bold black arrow. HDVRz and T7Φ represent the site of the Hepatitis delta virus ribozyme and the T7 terminator sequences, respectively.

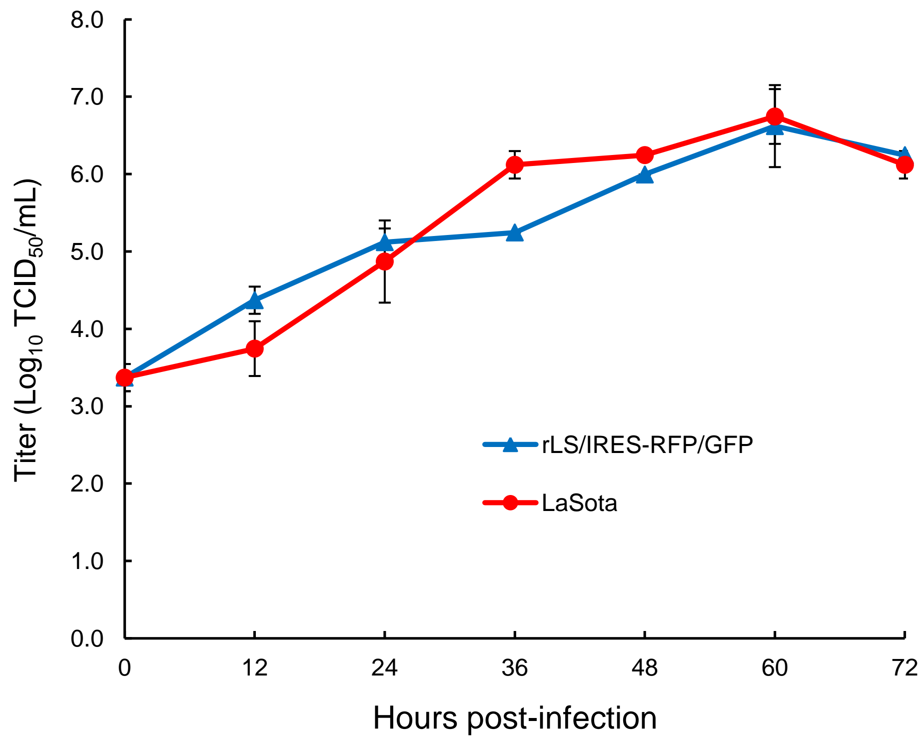

Fig. 2. Growth curve analysis comparing the viral replication of the rLS/IRES-RFP/GFP and parental LaSota viruses. DF-1 cells were infected with each virus separately at an MOI of 0.01. Every 12 h post-infection, virus lysates were harvested and subsequently, viral titers were determined by $TCID_{50}$ titration for each time point in DF-1 cells in triplicate. The mean titer of each time point of the two viruses is expressed in log_{10} $TCID_{50}$/ml.

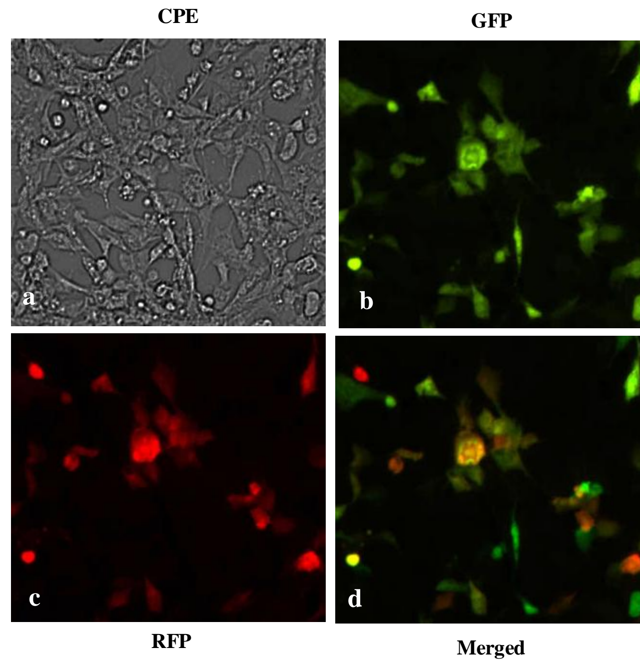

Fig. 3. Detection of RFP and GFP protein expression of the rLS/IRES-RFP/GFP virus by fluorescence microscopy. DF-1 cells were infected with rLS/IRES-RFP-GFP at an MOI of 0.01. At 24 h post-infection, infected cells were examined and viral CPE and corresponding fluorescence from the same field were digitally photographed at 100X magnifications with bright field (a) and GFP (b) and RFP (c) specific filter combinations (Nikon, Eclipse Ti, Melville, NY). The green and red fluorescent images (b and c) were merged (d).