

Rescue of recombinant Newcastle disease virus: current cloning strategies and RNA polymerase provision systems

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Received: 25 July 2016 / Accepted: 12 September 2016 / Published online: 1 October 2016
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Abstract Since the first rescue of a recombinant Newcastle disease virus (rNDV) in the late 1990s, many more rNDVs have been rescued by researchers around the world. Regardless of methodology, the main principle behind rescue of the virus has remained the same, i.e., the formation of a functional replication complex by simultaneously providing the full-length viral RNA and the viral NP, P and L proteins. However, different strategies have been reported for the insertion of the full-length genome into a suitable transcription vector, which remains the most challenging step of the rescue. Moreover, several systems have been published for provision of the DNA-dependent RNA polymerase, which is needed for transcription of viral RNA (vRNA) from the transfected plasmid DNA. The aim of this article is to consolidate all of the current cDNA assembly strategies and transcription systems used in rescue of rNDV in order to attain a better understanding of the advantages and disadvantages of each approach.

Abbreviations

NSV	Negative-sense virus
PSV	Positive-sense virus
NSNSV	Non-segmented negative-sense virus
NDV	Newcastle disease virus

vRNA Full-length viral RNA transcribed from plasmid DNA

Introduction

The process of generating recombinant viruses has come a long way. However, the generation of negative-sense RNA viruses (NSVs), either segmented (SNSV) or non-segmented (NSNSV), is more complex compared to the generation of recombinant DNA or positive-sense RNA viruses (PSVs) [52]. In the case of NSNSVs, this complexity is mainly due to the fact that the genomic RNA cannot be directly translated into viral proteins and is only functional when encapsidated within the NP protein, associated with the P and L proteins. This complex, called the ribonucleoprotein (RNP) complex, acts as a template for transcription and replication. Hence, for successful generation of any recombinant NSNSV (rNSNSV), formation of this complex in the cytoplasm of the host cell is an integral step in the process.

The first successful artificial formation of an RNP and subsequent generation of an rNSNSV completely from a cloned cDNA was reported using rabies virus (RABV) [56]. In 1999, Peeters et al. used the same principle to rescue the first rNDV from the lentogenic strain LaSota [49]. To date many different strains and types of NDV (lentogenic, mesogenic or velogenic) have been generated (Table 1). In all of these cases, the full-length genome cDNA is cloned into a plasmid vector known as a transcription vector, which possesses unique features that efficiently facilitate transcription of a viral RNA (vRNA) genome by an RNA polymerase of interest. In order to get a perfect vRNA with authentic 3' and 5' ends, the genomic cDNA must be cloned between the transcription initiation

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Table 1 Notable and selected studies on the rescue of rNDV, sorted by year published

Strain	Class	Genome length (nt)	Transcription vector	Copy number	HamRz	Final plasmid name	Assembly technique	Number of fragments	Gs after T7 promoter	RNA Pol. source	Pre or simultaneous infect/trans	Ref
LaSota	Lento	15,186	pOLTV5	Low	No	pNDPL	RE	7	GG	FPV-T7	Pre, 1 h	[49]
Clone-30	Lento	15,186	pX8/T	High	No	pflNDV-1	RE	5	GGG	BSR T7/5	N/A	[54]
Beaudette C	Meso	15,186	pBR322/dr	Low	No	pNDVf1	RE	8	GGG	MVA/T7	Simultaneous	[34]
LaSota	Lento	15,186	pBR322/dr	Low	No	pLaSota	RE	7	GGG	MVA/T7	Simultaneous	[25]
Hitchner B1	Lento	15,186	pSL1180	Low	No	pNDV/B1	RE	7	?	MVA/T7	Simultaneous	[46]
Mukteswar	Meso	15,186	pSL1180/CMV	Low	Yes	pNDVCMV	RE	5	GGG	Pol II	N/A	[38]
LaSota	Lento	15,186	pBluescript	Low	No	pFLC-LaSota	LJC	3	GGG	MVA/T7	Pre	[24]
NDV4-C	Aviru.	15,186	pOLTV6	Low	No	pY3-V4	RE	6	GG	plasmid	N/A	[65]
NDV4-C	Aviru	15,186	pCneo	High	Yes	pS-V4	RE	5	GG	Pol II	N/A	[65]
AF2240-I	Velo	15,192	pOLTV5-phiX	Low	No	pOLTV5-AF	Synthesis	1	None	BSR T7/5	N/A	UP
Strain	Class	Helper virus inf. rate	Cells	Transfection reagent	Helper vector	pNDV/pNP/pP/pL ratio	Clear sup. or lysate	Wash after transfect	Incubate/Inoculate (days)	ICPI	Ref	
LaSota	Lento	1 MOI	CEF/QM5	Fugene 6/Lipo	pCneo	0.25/0.4/0.2/0.2	Clear sup	Yes, 16 h	6/4	0.0	[49]	
Clone-30	Lento	N/A	BSR T7/5	Superfect	pCite 2a	5/2/2/1	Clear sup	?	3/5	0.25	[54]	
Beaudette C	Meso	1 FFA	HEP-2	Lipo Plus	pGEM-7Z	5/2.5/1.5/0.5	Clear sup	Yes, 12 h	8/2	1.63	[34]	
LaSota	Lento	1 pfu	HEP-2	Lipo Plus	pGEM-7Z	5/2.5/1.5/0.5	Clear sup	Yes, 4 h	6/4	0.29	[25]	
Hitchner B1	Lento	1 MOI	HEP-2	?	pTMI	1/0.4/0.2/0.2	Clear sup	?	4/4	?	[46]	
Mukteswar	Meso	N/A	BHK-21	Lipo	pCneo	1/2/1/1	Clear sup	Yes, 6 h	4/2	1.3	[38]	
LaSota	Lento	3 MOI	HEP-2	Lipo	pTMI	2/1/0.5/0.1	Lysate	Yes, 6 h	3/4	0.15	[24]	
NDV4-C	Aviru.	N/A	BHK-21	Lipo	pCneo	4/2/1/1	Clear sup	Yes, 6 h	4/?	0.0	[65]	
NDV4-C	Aviru	N/A	BSR T7/5	Lipo	pCneo	4/2/1/1	Clear sup	Yes, 6 h	4/?	0.0	[65]	
AF2240-I	Velo	N/A	BSR T7/5	Lipo	pCneo	0.25/0.4/0.2/0.2	Clear sup	Yes, 15 h	4/2	1.83	UP	

Note: all inoculations were in 9- to 11-day-old eggs. If different values are given in the reference, the maximum value is shown in the table. UP stands for unpublished data

Table 2 Sequence of the notable components of transcription vectors used in reverse genetics of mononegaviruses, including NDV

Name	Sequence
T7 promoter	TAATACGACTCACTATA[GGG]
Hammerhead ribozyme	GCGACTAGTTGTTAAGCGTCTGATGAGTCCGTGAGGACGAACTATAGGAAAGGAATTCCTATAGTC
HDV ribozyme	GGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCTGGGCATCCGAAGGAGGACGTCGTCCTCCACTCGGATGGC TAAGGGAGAGCTCG
T7 terminator	GATCCGGCTGCTAACAAAGCCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAA CCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTGGCTGAAAGGAGGAAGTATATCCGGATCGA

The names are sorted according to the location from 5' to 3'. Note: such vectors are made for transcription by T7 RNA polymerase. For RNA polymerase II systems, the T7 promoter and terminator are replaced with a CMV promoter and SV40 polyadenylation signals

site of the polymerase promoter and the autocatalytic cleavage site of a ribozyme without any extra nucleotides (see sections below). Helper genes of NP, P and L are separately cloned into expression vectors in tandem. Following co-transfection, the vRNA and the expressed proteins subsequently form the RNP complex, which leads to generation of new recombinant virus particles [56].

In the following sections, we review the most frequently employed, as well as the latest, methods of assembling the full-length cDNA within the transcription vector, as this step remains the most challenging of all the steps used for generation of the rNSNSVs. We also cover the latest innovations in provision and expression of RNA polymerase, which is essentially involved in fulfilling a key step in generating the vRNA from the full-length cDNA. For further background information on the structure, taxonomy, mechanism of transcription, and replication of NDV or other NSNSVs that are not covered in this review, the reader is referred to references [2, 15, 52].

Components of transcription vectors

Promoters

To allow transcription initiation for production of an authentic vRNA from a transfected plasmid DNA, promoter sequences are added to the 5' end of the antigenome. For transcription, T7 DNA-dependent RNA polymerase is often used, since it is highly processive and specifically recognizes a well-defined promoter sequence (-17 TAA-TACGACTCACTATAGGG +3). For efficient transcription, the natural GG or GGG nucleotides can be left right after the initiation site without significant consequences for the rescue efficiency. This is probably due to the unique regulation of transcription and replication of the paramyxoviruses (reviewed by Noton and Fearn, 2015 [48]).

Ribozymes

Self-cleaving RNAs, designated ribozymes, were first discovered in the early 1980s [8, 18, 35]. To date, many different origins and types of ribozymes have been identified. Use of ribozymes in the rescue of viruses is one of many applications for these RNA molecules. The sequences of ribozymes that are most often used as unique components for transcription vectors are shown in Table 2.

Hammerhead ribozyme

Several rNSNSV rescue systems, including those for RABV [27, 36], measles virus (MV) and borna disease virus (BDV) [42], employ an autocatalytic hammerhead sequence to process the 5' terminus of the full-length vRNA. Although such systems have been developed for some paramyxoviruses [64], including NDV [38, 65], they are not frequently used nor are they essential for the rescue of rNDV.

HDV ribozyme

Some viral ribozymes, such as the one derived from the hepatitis delta virus (HDV) RNA, provide autocatalytic cleavage at the 5' end of their own sequence. In 1994, Karl-Klaus Conzelmann and colleagues used this function to rescue the first rNSNSV, the strain SAD B19 of RABV, from its cloned cDNA [56]. They placed an 84-nt sequence of HDV ribozyme directly after the 5'-terminal non-coding sequence of the full-length RABV cDNA to generate the precise 3' end of the genome analogue after transcription from plasmid. To date, this technique has been used in the rescue of most rNSNSVs, with the HDV ribozyme (84-to-89 nt variants) being the most popular ribozyme in this technology. In most cases, rNSNSVs are then rescued by generating a T7/PolII-

generated full-length vRNA corresponding to the antigenome (i.e., the positive strand). The HDV ribozyme cleavage ensures the correct 3' end of the antigenome, which corresponds to the correct 5' end of the genomic RNA (i.e., (-)RNA).

Terminators

Lastly, a T7 terminator sequence must be located after the ribozyme sequence at the 3' end in order to correctly terminate transcription by the corresponding RNA polymerase. For the T7 RNA polymerase, the size of its terminator sequence is about 139 nt (Table 2), but it may vary depending on the system.

However, for systems using RNA polymerase II, the T7 promoter and terminator are replaced with the CMV promoter and SV40 early or late polyadenylation signals (see sections below).

Current methods of cloning the NDV antigenome into the transcription vector

Some of the studies described below may have already been reviewed in other articles, albeit in different capacities [26], but this current review focuses on gleaning a more in-depth methodology from those articles, which is necessary to effectively and coherently describe and compare techniques for assembly and cloning of the 15-kb antigenome into a transcription vector. In addition, new developments have been described recently [7, 24] and this article aims to cover all notable studies published to date.

Assembly

In view of the relative large size of the (-)ssRNA genome, cloning the full-length genome is no easy task. In fact, the full-length error-free cDNA assembly has remained the most challenging and elusive step, as reported over the last two decades. The efficiency of currently available commercial reverse transcription PCR (RT-PCR) systems is not sufficient to allow complete amplification of the whole genome in just one RT-PCR reaction. Moreover, since every NDV strain has its own unique sequence and naturally occurring restriction enzyme (RE) sites, the assembly requires a highly customized planning and design phase, and different from those published before. Cloning of the full-length antigenome into a specific kind of transcription vector becomes even more complicated when particular requirements, such as the rule-of-six [32, 50] and generation of the precise 3' and 5' ends [49, 56], have to be met. These challenges posed and specific know-how required have confined this technology to only a few laboratories.

Strategy one: sequential cloning based on naturally occurring RE sites in the genome

The sequential assembly of the full-length antigenome is the most popular method for the rescue of rNSNSVs, including rNDV. As mentioned above, the first successful rescue of an rNDV was reported back in 1999 [49]. After sequencing the RNA genome of the NDV strain LaSota, a lentogenic vaccine strain that is used worldwide, Peeters and colleagues generated a low-copy-number transcription vector named pOLTV5 by combining the pOK12 and transcription vector 2.0 plasmids to feature unique *StuI* and *SmaI* RE sites located between the T7 promoter and the HDV ribozyme sequence. Hence, any fragment cloned between these two sites could be directly transcribed by the T7 RNA polymerase. In the first cloning step, two short PCR products corresponding to the exact 3'- or 5'-terminal ends of NDV were joined in an overlap-extension PCR (OE-PCR) and cloned between the two RE sites. Next, the full-length NDV antigenome was assembled within the plasmid by joining the remaining PCR-amplified subgenomic fragments at overlaps using shared RE sites [49]. Meanwhile, the NP, P and L genes were also cloned into pCIneo, an expression plasmid containing the CMV promoter. Subsequently, all plasmids were co-transfected and the recombinant virus was recovered (see “[System one: recombinant T7-expressing poxviruses](#)”).

The second successful rescue, published almost simultaneously with the first report, was reported with a few differences [54]. Römer-Oberdörfer and colleagues succeeded in assembling the full-length NDV strain Clone-30 cDNA in the *SmaI* RE site of pX8 δ T, which was the same plasmid used by Conzelmann and colleagues in the rescue of rRABV in 1994 [56]. cDNA was reverse transcribed from the leader to trailer using three primers. The first one was used to synthesize a nearly 8-kb fragment; the second one, a nearly 4-kb fragment; and the third one, a 3-kb fragment. Five fragments were amplified from the cDNA fragments, two of which were short PCR products covering the leader and trailer, with both having artificial *MluI* sites. First, the short fragments were cloned into pX8 δ T and joined through the *MluI* site. Next, the remaining three fragments were joined using naturally occurring *BsiW* and *StuI* RE sites while having another pair of artificial *MluI* at both ends of the combined fragment. This fragment was then cloned between the short fragments to form the full-length NDV genome.

The third rescue was reported by Samal and colleagues a few months later in 2000 [34]. The low-copy-number pBR322 plasmid was modified to include a 72-nt linker between *EcoRI* and *PstI* RE sites and was thus renamed pBR322/dr. Eight PCR products were generated, covering the whole genome of NDV strain Beaudette C. However, unlike the first two systems, the fragments containing the

leader and trailer were not short. In a series of cloning procedures, all fragments were joined using naturally occurring or artificial RE sites to form the full-length antigenome between the T7 promoter and the HDV ribozyme sequence.

The three assembly methods described above can be considered the original methods for rNDV rescue, with each being cited frequently since their publication within a 2-year period between 1999 and 2000. Interestingly, the rescue approaches and the RNA polymerase provision systems in these reports were not entirely similar (see “[Transcription and rescue; RNA polymerase systems](#)”). Nonetheless, each group produced at least one more publication on rNDV [25, 43, 50, 51] before a group led by Garcia-Sastre and Palese succeeded in rescuing the lentogenic NDV strain Hitchner B1 by assembling the antigenome into the low-copy number pSL1180 [46]. In their report, seven fragments were joined mostly through naturally occurring RE sites, with two additional RE sites of *SacII* and *XbaI* introduced between the NP-P and P-M intergenic sequences, respectively, to function as genetic tags.

The other rNDV rescue systems that subsequently followed are mostly based on the systems described above or have used the concept developed by Conzelmann and colleagues in 1994 [56].

As seen above, the order of cloning of the subgenomic fragments is unrelated to the order of genes or distance to the terminal ends. In fact, it is dependent entirely on the unique genome sequence of a particular strain and the naturally occurring RE sites; the strategy selected will be primarily determined by the available sequences. Therefore, strategy determination is extremely crucial and requires meticulous attention. In the sections below, we review alternative and simplified methods that have been developed for rapid assembly of the rNDV antigenome.

Strategy two: ligation-independent cloning

Ligation-independent cloning (LIC) systems that do not require the use of REs have been developed [1], sometimes, with slight adjustments/differences [11, 40, 41]. In fact, assembly of the antigenome of NDV using an In-Fusion[®] PCR (Clontech, USA) system has been reported frequently in recent years [7, 9, 24, 39, 63, 67–69]. Using this LIC method, cloning of the entire full-length positive-sense antigenome of NDV is possible within a few weeks, offering a rapid, versatile and reliable approach compared to the traditional assembly of DNA fragments using the RE strategy [68].

The first report describing the In-Fusion[®] PCR cloning system for rescue of rNDV was published in 2011. Hu et al. used this RE-independent strategy to create three cDNA

fragments spanning the entire genome of the LaSota strain, each approximately 5 kbp in length [24]. All three fragments were amplified using primers that contained a 15-nt overlapping region of homology (to the vector or another insert) at their 5' end as required by the system. These fragments were then sequentially assembled into a modified low-copy-number pBluescript plasmid, which was linearized by PCR using a pair of specific primers before each phase of the In-Fusion[®] reaction. The final plasmid, designated pFLC-LaSota, was then introduced into Stb12 competent cells by transformation as recommended by the manufacturer. pFLC-LaSota is the first plasmid of its kind to be generated by the LIC method. Stellar competent cells are usually recommended for transformation of ligation reactions in this system.

The In-Fusion[®] systems have also been used for easy and efficient insertion of foreign genes into the antigenome. The G gene of aMPV-A (UK, 14/1), -B (Hungary, 567/4) or -C (Colorado) was inserted between the F and HN genes of the pFLC-LaSota vector using the In-Fusion[®] system to study host immune responses as well as protection against challenge [24, 63]. In a separate study, insertion of a red fluorescent protein (RFP) between the F and HN genes for co-infection studies using the same LIC system has been reported [39].

In another related study, a plasmid containing the minigenome of NDV strain VG/GA was generated using this system [68]. The LIC-generated minigenome was rescued within just three weeks. In addition, a separate study was reported in which a novel system was developed for expression of foreign genes by NDV from a second ORF through an internal ribosomal entry site (IRES), using the In-Fusion[®] system [66].

OE-PCR [23, 62] could also be considered an alternative LIC method for generation of the full-length antigenome. In fact, Murulitharan et al. (personal communication) attempted to generate the entire NDV antigenome in just a few days, by OE-PCR. Four PCR-amplified fragments were generated using primers specifically designed to produce approximately 20 bp of complementary sequences at the 3' ends. The experimental design involved the use of four PCR-amplified fragments as templates for the subsequent PCRs with Phusion polymerase [5] without the use of any primers for the initial 15–20 cycles, following which the 5'-end primers were added and the PCR was continued by maintaining the annealing temperature at 60 °C. Unfortunately, the attempt was unsuccessful due to a reduction in DNA concentration of the gel-purified bands, making the subsequent fourth and final PCR less robust. This resulted in the amplification of only 12 kb out of the 15 kb of the genome. However, with an improved protocol, reagents and materials, the replication of this

method could potentially culminate in the production of custom-made viral genomes in a few quick steps compared to the RE assembly method.

Strategy three: synthetic production

The unique design of some plasmids, such as the high-copy-number pTVT7R-phiX [30] and the low-copy-number pOLTV5-phiX (Murulitharan et al. 2016, unpublished data), allows the insertion of the whole positive-sense antigenome in just one cloning step. pOLTV5-phiX is made by transferring a *SapI*-*Bam*HI fragment from pTVT7R-phiX containing a bacteriophage phiX174 fragment to the low-copy-number pOLTV5 [49]. The presence of *Bbs*I sites on the plasmid and addition of *Bsm*BI sites to the 3' and 5' ends of the positive-strand genome will result in incorporation of the full-length antigenome right between the T7 promoter and HDV ribozyme sites (Fig. 1). Digestion with *Bbs*I releases a visible 674-nt fragment containing the phiX fragment after agarose gel electrophoresis, hence its advantage over its parental plasmid pTVT7R (0.0) [29]. At the same time, new advances in chemical synthesis of long nucleic acid fragments allow the production of a completely synthetic positive-stranded NDV genome, with additional *Bsm*BI sites and few overhanging nucleotides, to facilitate rapid ligation into the pOLTV5-phiX transcription vector. If there are any *Bsm*BI sites present within the genome, it should be modified to avoid digestion of the genome with the enzyme intended to digest the 3' and 5' ends. Murulitharan and colleagues replaced the "G" with an "A" (CGTCTC to CATCTC) to eliminate the naturally occurring *Bsm*BI site in the NDV strain AF2240-I (Murulitharan et al. 2016, unpublished data). With respect to the ORF, this change did not affect the amino acid produced, and the substitution could also be used as a marker. In addition, the use of pOLTV5-phiX allowed for optional addition of "GG" or "GGG" at the end of the T7 promoter (Murulitharan et al. 2016, unpublished data).

Advantages and disadvantages of each strategy

Although the sequential cloning method using the naturally occurring RE sites has been widely used in rescue of rNSNSVs, several drawbacks are associated with this strategy [68]. Most importantly, the sequential cloning process is extremely time-consuming, taking months at a time to complete (Table 3). Moreover, unique naturally occurring RE sites may be located in unsuitable areas of the genome. Furthermore, ligation and transformation of large DNA fragments are not always efficient. In fact, in order to make ligation easier, synthesis of shorter DNA fragments has been frequently reported [49, 54, 56]. Furthermore, sometimes the use of REs that generate blunt ends may complicate the ligation of large fragments even more.

Therefore, for assembly of the full-length cDNA, the RE strategy requires a high level of expertise in genetic engineering and molecular cloning to be accomplished [68]. Moreover, when a vaccine is urgently needed, this method lacks practicality due to its laborious process.

Murulitharan et al. have overcome the drawbacks of this method by developing rapid synthesis and cloning of the genome cDNA for a viscerotropic velogenic NDV strain AF2240-I within just a few weeks (personal communication, unpublished data). As shown in Fig. 1, if the extra nucleotides and the *Bsm*BI sites were carefully added to the genome cDNA, this method could be employed for the recovery of the other NDV strains. However, several considerations, such as the reproducibility of the protocol, the specificity of the recovered recombinant virus, and its genetic stability, should be taken into account. Yet, synthetic synthesis of the antigenome, followed by rapid cloning into a suitable transcription vector, could be a powerful tool in the development of NSNSV-based recombinant viruses for the purpose of gene therapy or production of recombinant vaccines.

Transcription and rescue: RNA polymerase systems

During the rescue of an rNDV, an RNA polymerase enzyme needs to be present to transcribe the vRNA from the transcription vector [49]. Once the viral genomic RNA and mRNAs have been synthesized, they will take over, and subsequently, virus replication will ensue as in a natural infection. Rescue systems using the bacteriophage T7 RNA polymerase have been widely used. The T7 RNA polymerase allows for cytoplasmic transcription of vRNAs, thus mimicking cytoplasmic-replicating viruses [2]. Several other systems have also been developed, which will be described in the sections below.

One of the key differences between the rescue systems for rNDV and those for other rNSNSVs is that the lentogenic rNDV requires cleavage of the F0 (fusion) protein. Cleavage of the F0 protein is required for cell-to-cell spread and the generation of an infectious virus. Therefore, inoculation into the allantoic cavity of embryonated eggs is often used for the rescue of lentogenic rNDV, since most cell lines lack trypsin-like proteases required for efficient cleavage of the F0 protein [17, 45, 55].

Popular systems

System one: recombinant T7-expressing poxviruses

This system is one of the two most popular rescue systems, not only for rNDV but also the other rNSNSVs. In this

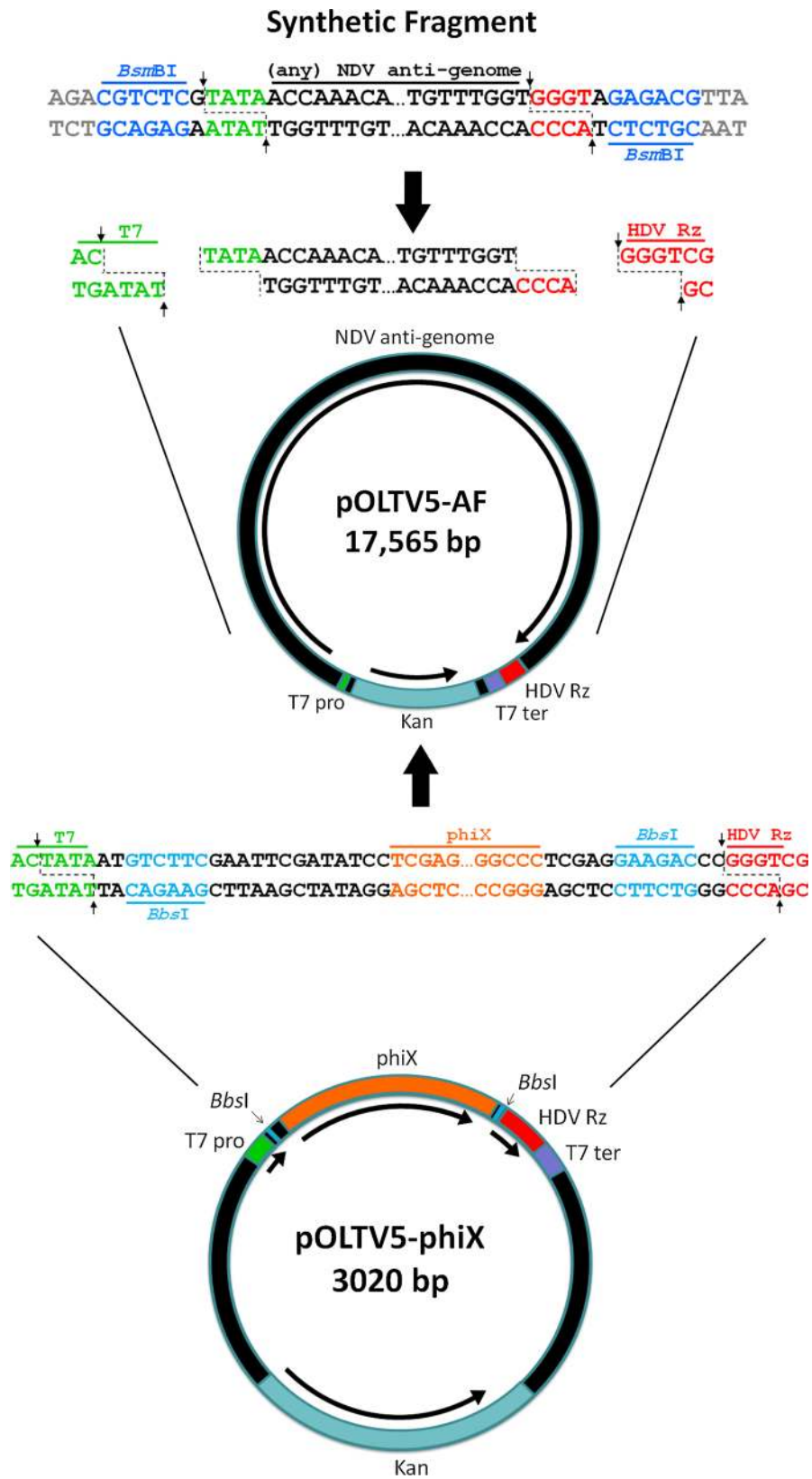


Fig. 1 A schematic diagram showing the ability of pOLTV5-phiX to be cloned with a synthesized NDV antigenome. *BbsI* and *BsmBI* digestions leave matching sticky ends on both sides of the antigenome

and the plasmid. Any NSNSV antigenome having the additional sequences shown above could be directly and efficiently cloned into the plasmid

Table 3 A comparison between cost and time of preparation of a cloned genomic cDNA based on different assembly strategies

Method	Preparation	Genome sequencing	Plan and design	PCR	Cloning	Total cost	Time	Advantage or disadvantage
SC	2 w - 1 m	1 d - 2 w	1 d - 1 w	1 d - 1 w	1 m	<\$10 k	2.5-3 m	Slow, multiple cloning
OE-PCR	2 w - 1 m	1 d - 2 w	1 d - 1 w	1 w	1 w	<\$10 k	2-2.5 m	Semi-fast
Synthesis	2 w - 1 m	1 d - 2 w	1 d	-	1 w	\$10-15 k	1-1.5 m	Fast

Note: Preparations include plaque assay, inoculation, harvest, and purification but do not include field isolation and classification. Genome sequencing may vary depending on NGS or conventional sequencing techniques used. Cloning includes digestion, ligation, transformation and plasmid extraction. Total cost is an estimate and may vary depending on different factors; only the costs of reagents and synthesis are considered, and the costs of lab equipment, instruments or sequencing are excluded. SC, sequential cloning; OE-PCR, overlap extension PCR; d, day; w, week; m, month

system, cells are infected with a T7-expressing poxvirus (either vTF7-3, MVA-T7 or FPV-T7) prior to or during transfection (see Table 1). The recombinant poxvirus is a DNA virus that replicates in the cytoplasm and is genetically engineered to carry the bacteriophage T7 RNA polymerase gene.

The recombinant vaccinia virus vTF7-3 [14] was first used by Conzelmann's group for the rescue of rRABV in 1994 [56], but in the rescue of rNDV, an engineered, highly-attenuated, host-range-restricted modified vaccinia virus Ankara (MVA/T7) is usually used [57]. The modified MVA/T7 is unable to multiply in most mammalian cells [57], but it can replicate in avian cells such as CEF. Interestingly, MVA/T7 had not been used in the first two rescues of rNDV and only became mainstream after the third rNDV was generated in the year 2000 [34].

In this system, infection of cells with MVA/T7 prior to or simultaneously with transfection has been reported (Table 1). Nonetheless, the infection rate is usually kept at a multiplicity of infection (MOI) of 1 to 3. In most protocols, the supernatant is replaced with fresh medium a few hours after infection/transfection. After a few days, 100-300 μ l of a briefly centrifuged and cleared supernatant is injected into the allantoic fluid of 9- to 11-day-old embryonated SPF chicken eggs. To obtain a higher yield, viruses in the supernatant are sometimes further amplified in the same or other cell lines prior to injection into the allantoic fluid [34]. However, this is not applicable to the lentogenic rNDV unless trypsin is used. Hep-2 cells have often been used in this system (Table 1), mostly because they are permissive for NDV. They are also transfected efficiently and have been shown to be resistant to CPE caused by MVA.

The recombinant fowlpox virus expressing T7 RNA polymerase, FPV-T7, was made by recombination between pEFLT7PolC1-6 containing the T7 gene and wtFPV in CEF cells [4]. Although the use of FPV-T7 in the rescue of rNDV has not been as frequent as the other methods in recent years, it should be noted that the first rNDV was recovered using the FPV-T7 system [49]; following successful insertion of the NDV antigenome into pOLTV5,

Peeters and colleagues infected CEF or QM5 cells with FPV-T7 at an MOI of 1 for 1 h at 37 °C. Subsequently, the cells were co-transfected with the four plasmids, and after incubation for 4 h (CEF cells) or 16 h (QM5 cells), the transfection mixture was replaced by culture medium containing 5 % allantoic fluid in order to provide the essential proteases for the cleavage of the F protein. Three to six days later, the culture supernatant was harvested and inoculated into embryonated SPF eggs.

System two: cell lines stably expressing T7 RNA polymerase

The generation of several T7-expressing cell lines has been reported in the last few decades [10, 58, 70]. These cells have been used for different applications, one of which is to rescue viruses. Among all of the engineered cell lines, BSRT7/5 cells generated by Conzelmann's group in 1999 remain the most widely used cell line for rescue of the rNSNSVs [6]. Römer-Oberdörfer and colleagues were the first to use this cell line specifically for the rescue of rNDV [54]. Since then, the approach has been adopted by many other groups [9, 38, 65].

As recommended by the original developers of BSRT7/5 (personal conversation), the cells must be maintained in Glasgow's minimum essential medium (GMEM) supplemented with 10 % newborn calf serum (NCS), tryptone phosphate, MEM amino acids and antibiotics. Addition of geneticin (G418) in every second passage for selection of the T7-expressing cells is essential. Furthermore, although the recommended protocol for transfection of the cell line uses calcium phosphate (CaPO₄) transfection [6, 44], the use of reagents such as Lipofectamine has also been reported [38]. Other than that, the protocol for transfection is similar to those of other cells that are permissive for NDV replication. Briefly, cells are grown to a confluency of 80 % before being transfected with a plasmid mixture of NP, P, L and the full-length cDNA. After a few days of incubation, the supernatant is used for inoculation into SPF eggs [54] (Table 1).

System three: plasmid-based expression of T7 RNA polymerase

Plasmids expressing T7 RNA polymerase have also been widely used in the rescue of paramyxoviruses [13, 20, 42, 60], rNSNSVs [42, 47, 60], rNSVs [37] or other viruses. Plasmids such as pAR3126 [20], pCMV/T7-T7pol [3], pC-T7pol [47], pCA-T7 [42], pCMV-T7 [60] and pADT7(N) Δ pT7 [31] are potential candidates that have been shown to be effective in the rescue of many viruses, including paramyxoviruses. To optimize protein expression by these pT7 plasmids, inclusion of a Kozak consensus sequence [33] at the 5' of the initiator ATG is recommended [31, 60]. In plasmids such as pCA-T7 and pCAGGS/T7, expression of the T7 RNA polymerase comes under the control of the chicken β -actin promoter [21, 42], while in others it is under the control of an RNA polymerase II promoter such as the CMV promoter [37]. Furthermore, it has been reported that plasmids with a chicken β -actin promoter display higher expression levels for the T7 RNA polymerase than those with a CMV promoter, and different cell lines can be used for rescue [13]. In addition, in some cases, such as pADT7(N) Δ pT7, the T7 promoter is inactivated so that expression of the T7 RNA polymerase is solely driven by the CMV promoter [31].

Establishment of a pT7-driven minigenome system for NDV was first reported in 2009 [28]. Jiang and colleagues constructed a minigenome of NDV strain V4, a few hundred bp in length, containing the leader and trailer regions of the genome cloned into a low-copy pBR plasmid. To visualize the generation of virus particles, a reporter EGFP gene was inserted within the fragment, and the plasmid was called pBR-EGFP. Next, BHK-21 cells were transfected with pCAGGS/T7, pBR-EGFP, and helper plasmids containing the NP, P and L genes of LaSota strain at a ratio of 2:2:2:1:1. Green fluorescence signals were observed after 48 h, indicating successful replication and expression of the minigenome. Four years later in 2013, a plasmid-driven T7 RNA polymerase was used for the rescue of a complete particle of rNDV from the full-length antigenome [65]; BHK-21 cells were co-transfected with 5 μ g of plasmid pCAGGS/T7 and a plasmid containing the antigenome of an avirulent and thermostable (heat-resistant) NDV4-C strain and helper genes derived from the same strain cloned in pCIneo. After a typical wash with PBS at 6 h post-transfection, the cells were maintained in Opti-MEM containing 1 μ g of N-tosyl-phenylalanine chloromethyl ketone (TPCK)-treated trypsin per ml for 4 days until the supernatant was inoculated into SPF eggs.

System four: RNA polymerase II as a non-T7 RNA polymerase system

rNSNSV reverse genetic systems that work independently from the conventional T7 RNA polymerase system have been developed [27, 38, 42, 59, 65]. According to these reports, higher levels of RABV, MV and NDV vRNA transcription were achieved when an RNA polymerase II promoter was used instead of the T7 RNA polymerase promoter [27, 42]. RABV and MV have a cytoplasmic replication strategy, similar to that of NDV. Therefore, when a CMV promoter is located upstream of the antigenome, transcription is driven by the cellular RNA polymerase II.

The use of this approach in the rescue of rNDV has been shown to be effective in recovery of a higher-titer virus [38, 59, 65], and such advances will consequently lead to improvements in the reverse genetics of NDV. Li et al. used a 670-nt portion of this sequence [38], whereas others [59, 65] used a 750-nt enhancer/promoter of CMV, which was already available in the pCIneo plasmid. Interestingly, in most cases, a hammerhead ribozyme has been located between the CMV promoter and the NDV leader sequence.

One other important feature of this approach is the addition of SV40 early [38] or late [59, 65] transcription-termination signals directly after the HDV ribozyme sequence. Since the SV40 late polyadenylation signal has been shown to have a greater efficiency than the SV40 early polyadenylation signal, the former is probably the best choice.

Pros and cons of different transcription systems

In “[System one: recombinant T7-expressing poxviruses](#)”, recombinant T7-expressing poxviruses were reviewed. It should be noted that FPV-T7 is an interesting alternative to the widely used vTF7-3 or MVA/T7 systems [4]. FPV, a member of the genus *Avipoxvirus* of the family *Poxviridae*, is an avian virus that is non-infectious for mammalian species and is less cytotoxic than MVA-T7 or vTF7-3. Because it is not infectious, handling of this virus requires less stringent microbiological safety conditions.

T7-expressing cells were generated to avoid cytotoxicity and the risk of contamination of rescued viruses by T7-expressing helper viruses. Hence, one of the advantages of using the BSRT7/5 cell line is that the pre- or simultaneous infection with a helper virus is not required. Instead, plasmids can be introduced directly into the cells by transfection at the concentrations and ratios shown in Table 1. Moreover, the deficiency in the RIG-I pathway of

BHK-21 and BSRT7/5 cells results in less interferon induction, thus making the rescue more efficient [19].

In 1995, Radecke et al. reported the generation of an engineered 293 cell line that stably expressed the N and P helper proteins of MV virus as well as the T7 RNA polymerase [53]. The cell line was used to generate rMV from the cloned cDNA. Clearly, using such systems, the number of rNSV helper plasmids used during co-transfection would be markedly reduced. However, several drawbacks may be associated with these systems. For example, expression of the T7 RNA polymerase is not always sufficient to lead to efficient transcription of vRNA [38, 47]. Moreover, not all cell lines are easy to validate when producing a vaccine [60], not to mention difficult to develop and maintain.

In general, although the T7-expressing viruses and cells are widely used, neither is ideal for viral vaccine development [60]. Replication of the T7-expressing viruses during rescue may cause a cytopathic effect (CPE) in cells and interfere with propagation of rNDV. In addition, other drawbacks include contamination of the newly generated rNDV with the T7-virus, requiring a stringent purification step to remove all traces of the helper virus. Moreover, in cells infected with poxviruses, including vaccinia and fowlpox viruses, there is a strong possibility of recombination between plasmids [12, 16, 22]. The T7-expressing vaccinia virus may also cause impaired processing of viral genes and interfere with efficient rescue [37]. Finally, some recombinant viruses such as MVA shut off protein synthesis of the host cell, which may have additional unintended effects on cellular processes [61]. These are a few reasons why other methods for provision of the RNA polymerase, e.g., plasmid-based T7 RNA polymerase or transcription by RNA polymerase II, are sometimes preferred. For a complete review of such systems, the reader is referred to reference 2.

Summary

In this review, we have summarized the current approaches for cloning the full-length NDV cDNA and transcription systems for generating vRNA. In addition, we have analyzed the advantages and disadvantages of each approach to provide insight into selecting appropriate strategies and transcription systems. Table 3 provides a comparison between assembly strategies. Nonetheless, it is observed that the RE site strategy is by far the most used method. In closing, transcription systems have also been improved in recent years, providing less contamination and toxicity during rescue, thus holding many promises for future studies on the development of an effective rNDV vaccine.

Acknowledgments The authors would like to thank Erin Lim for comprehensive editing of the manuscript.

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