Video Article

Reverse Genetics Mediated Recovery of Infectious Murine Norovirus

Armando Arias*[1], Luis Ureña*[1], Lucy Thorne[1], Muhammad A. Yunus[1], Ian Goodfellow[1]

[1]Section of Virology, Imperial College London

*These authors contributed equally

Correspondence to: Armando Arias at a.arias-esteban@imperial.ac.uk, Ian Goodfellow at i.goodfellow@imperial.ac.uk

URL: https://www.jove.com/video/4145
DOI: doi:10.3791/4145

Keywords: Virology, Issue 64, Immunology, Genetics, Infection, RNA virus, VPg, RNA capping, T7 RNA polymerase, calicivirus, norovirus

Date Published: 6/24/2012

Citation: Arias, A., Ureña, L., Thorne, L., Yunus, M.A., Goodfellow, I. Reverse Genetics Mediated Recovery of Infectious Murine Norovirus. *J. Vis. Exp.* (64), e4145, doi:10.3791/4145 (2012).

Abstract

Human noroviruses are responsible for most cases of human gastroenteritis (GE) worldwide and are recurrent problem in environments where close person-to-person contact cannot be avoided [1, 2]. During the last few years an increase in the incidence of outbreaks in hospitals has been reported, causing significant disruptions to their operational capacity as well as large economic losses. The identification of new antiviral approaches has been limited due to the inability of human noroviruses to complete a productive infection in cell culture [3]. The recent isolation of a murine norovirus (MNV), closely related to human norovirus [4] but which can be propagated in cells [5] has opened new avenues for the investigation of these pathogens [6, 7].

MNV replication results in the synthesis of new positive sense genomic and subgenomic RNA molecules, the latter of which corresponds to the last third of the viral genome (**Figure 1**). MNV contains four different open reading frames (ORFs), of which ORF1 occupies most of the genome and encodes seven non-structural proteins (NS1-7) released from a polyprotein precursor. ORF2 and ORF3 are contained within the subgenomic RNA region and encode the capsid proteins (VP1 and VP2, respectively) (**Figure 1**). Recently, we have identified that additional ORF4 overlapping ORF2 but in a different reading frame is functional and encodes for a mitochondrial localised virulence factor (VF1) [8].

Replication for positive sense RNA viruses, including noroviruses, takes place in the cytoplasm resulting in the synthesis of new uncapped RNA genomes. To promote viral translation, viruses exploit different strategies aimed at recruiting the cellular protein synthesis machinery [9-11]. Interestingly, norovirus translation is driven by the multifunctional viral protein-primer VPg covalently linked to the 5' end of both genomic and subgenomic RNAs [12-14]. This sophisticated mechanism of translation is likely to be a major factor in the limited efficiency of viral recovery by conventional reverse genetics approaches.

Here we report two different strategies based on the generation of murine norovirus-1 (referred to as MNV herewith) transcripts capped at the 5' end. One of the methods involves both *in vitro* synthesis and capping of viral RNA, whereas the second approach entails the transcription of MNV cDNA in cells expressing T7 RNA polymerase. The availability of these reverse genetics systems for the study of MNV and a small animal model has provided an unprecedented ability to dissect the role of viral sequences in replication and pathogenesis [15-17].

Video Link

The video component of this article can be found at https://www.jove.com/video/4145/

Protocol

1. RNA Transcription and Capping for The Recovery of Infectious MNV

This protocol is designed to allow the efficient recovery of infectious MNV from cDNA via *in vitro* transcription and its subsequent *in vitro* capping (section 1.1). The resulting capped transcripts are then transfected into cells to recover infectious MNV (sections 1.2 and 1.3). This approach provides the most sensitive method for the recovery of MNV with typical yields in excess of 10^5 infectious units per 35 mm (in diameter)-dish of cells for MNV. The protocol is detailed below:

1.1 Synthesis of infectious capped MNV transcripts:

1. Digest the plasmid containing the wild type MNV cDNA (pT7:MNV 3'Rz) with *Nhe*I to obtain linear DNA. *Nhe*I recognises a unique restriction site after the 3' end polyA tail of MNV genome (**Figure 2**). Linearised plasmids are typically purified with using silica columns (e.g. GFX *PCR DNA Gel Band Purification Kit* from GE Healthcare) and eluted in H_2O.
2. *In vitro* transcribe the linearised vector using T7 RNA polymerase as previously described [17]. Many commercial kits are available for this purpose and provide a reproducible method of large amounts of RNA synthesis such as MEGAScript (Life Technologies) and RiboMAX (Promega). Transcription reactions are typically DNAse digested prior to further analysis; however in many instances this is not required as lithium chloride purifications as described below do not precipitate DNA efficiently.

3. Analyze a small aliquot of the RNA transcription reaction, typically 0.5 µl or less, by agarose gel electrophoresis to ensure the transcription reaction has worked efficiently and RNA is full-length. Whilst many users may want to run denaturing gels to correctly size the RNA, we typically use non-denaturing agarose gel electrophoresis as a rapid method to analyse RNA integrity. The MNV genome as produced from the infectious cDNA clone pT7:MNV 3'Rz will run at approximately 3 Kbp relative to a dsDNA ladder on a non-denaturing agarose gel (**Figure 3**).
4. Consider that other methods are available as an alternative to obtain a fast analysis of RNA integrity such as using an Agilent bioanalyser (**Figure 3**).
5. Note that poor gel resolution may be encountered if too much RNA is loaded. Take great care to ensure the agarose gel is prepared using RNAse-free reagents to avoid RNA degradation during electrophoresis which may affect band resolution. Heating RNA to 65 °C followed by cooling on ice may also help in some instances.
6. Purify RNA sample to remove the unincorporated nucleotides. Many methods are available for this including silica column based approaches however in this protocol we typically use lithium chloride as a cost effective alternative. For this purpose, add H_2O to reach a final volume of 100 µl and then add 40 µl of *Lithium chloride precipitation solution* (7.5 M LiCl, 50 mM EDTA, pH 8.0, Ambion) and store the sample at -20 °C for at least 30 min.
7. Pellet the RNA by centrifugation at 12,000 X *g* at 4 °C for 15 min.
8. Remove the supernatant, taking care not to disturb the translucent RNA pellet and wash it in 150 µl of 70% ethanol. Centrifuge the tube at 12,000 X *g* at 4 °C for 15 min.
9. Remove the ethanol and air-dry the RNA, avoiding the pellet to completely dry out as this will make resuspension difficult.
10. Then, resuspend the MNV transcripts into 50-100 µl of *RNA storage solution* (Ambion). Care should be taken to ensure all the RNA has dissolved properly. Should the RNA appear difficult to dissolve fully, heating the sample to 60 °C may help to resuspend it. Any insoluble material should then be removed by centrifugation prior to RNA quantification. The purified transcripts are uncapped and require a subsequent *in vitro* capping step to be infectious (ScriptCap m7G Capping System, Epicentre Biotechnologies).
11. Quantify the RNA by spectrophotometry. Depending on the nature and scale of the transcription reaction, typical yields range from 50-150 µg of RNA per 100 µl transcription reaction. The integrity of RNA should be analysed before the capping reaction by running out 100-300 ng of sample into a 1% agarose gel (**Figure 3**).
12. To improve the efficiency of RNA capping, heat 60 to 70 µg of MNV RNA transcripts at 65 °C for 10 min and then place the tube immediately on ice. This step may reduce any inhibitory effect of RNA structure on the capping. Pulse the tube in a chilled microfuge to collect droplets formed during the heating step.
13. Prepare a capping reaction mixture as suggested by the manufacturer (*ScriptCap m7G Capping System*, Epicentre Biotechnologies). Briefly, add 60-70 µg of MNV RNA to a final reaction volume of 100 µl. The capping reaction mix may contain 10 µl of 10 x Capping buffer (500 mM TrisHCl pH 8.0, 60 mM KCl, 12.5 mM MgCl2), 10 µl of 10 mM GTP, 0.5 µl of 20 mM S-adenosyl methionine, 2.5 µl of Scriptguard (100 units), and 4 µl of Scriptcap enzyme (40 units).
14. During the reaction set up, keep RNA transcripts on ice to avoid degradation. Mix well the reaction mixture and then incubate it at 37 °C for 1 h. Note the reaction size may be scaled according to the amount of capped transcript required.
15. Purify the RNA by LiCl precipitation as explained above (see point 1.1.6). Dissolve the pellet in 50-100 µl of RNA storage solution (Ambion) and quantify the amount of RNA. Typically, RNA samples are subsequently normalised to 1 µg/µl. Again, check all the RNA has dissolved properly. If it has not been properly dissolved, heat the sample to 60 °C to allow its dissolution. Remove by centrifugation any insoluble material prior to RNA quantification.
16. Check the integrity of RNA again before proceeding with the transfection step. To this aim, run an amount of 100-300 ng of sample into a 1% agarose gel (**Figure 3**).

1.2 Recovery by Neon-mediated transfection of RNA into Raw264.7 cells:

For the recovery of MNV infectious virions in a permissive cell line it is possible to electroporate the capped MNV transcript into Raw264.7 cells using *Neon transfection* system (Invitrogen). Raw264.7 are cells susceptible to MNV infection, supporting multiple rounds of virus replication and subsequent re-infection. As a result, typical yields will approach in excess of 10^5 infectious units per ml at 24 hours post transfection but peak at $>10^7$ infectious units after 48 hours.

1. One day before transfection, seed Raw264.7 cells at an estimated 50% confluency. Typically two T75 flasks of cells are required for 3 transfections.
2. The day of transfection, scrape the cell monolayers into Dulbecco's modified Eagle medium (DMEM) containing 10% foetal calf serum (FCS), ensuring you generate a single cell suspension by repeated pipetting.
3. Determine the concentration of viable cells in a haemocytometer using trypan blue exclusion to label the non-viable cells.
4. Pellet the cells at 1,200 X *g* for 5 min and resuspend them in DMEM containing 10% FCS at a final concentration of 8 x 10^6 cells/ml.
5. Just prior to transfection, aliquot 1 ml of cells per transfection and pellet them at 1,200 X *g* for 2 min. Remove the media and wash the cells in 500 µl of PBS (without Mg^{2+}/Ca^{2+}). Spin down the cells again at 1,200 X *g* for 2 min. Note it is advisable to keep cells in DMEM for as long as possible as storage in PBS for long periods of time may compromise the cell viability and transfection rate.
6. Remove PBS from tubes and add 130 µl of resuspension solution *(Neon transfection system kit*, Invitrogen) to a final concentration of 6 x 10^7 cells/ml. Care should be taken to resuspend the cells avoiding the formation of bubbles which will cause sparking during transfection and compromise cell survival.
7. Add the appropriate amount of capped MNV transcript to the cells (**Figure 2**), generally 1.3 µg of capped RNA is added to 130 µl of resuspended cells and mixed gently. Then, collect 100 µl of the mixture in the 100 µl Neon transfection tip. Special care should be taken to ensure that no bubbles are formed in the electroporation cuvette (*Neon transfection* system kit 100 µl tip) as this will cause failure of the experiment.
8. Electroporate the cells using a single pulse at 1,700V for 25 msec, ensuring the absence of sparks during pulsing which will indicate the presence of bubbles in the sample. In the case sparking should occur, discard the sample and repeat the transfection. Release the cells into an Eppendorf tube containing 1 ml of antibiotic free DMEM containing 10% FCS. Note each tip can be reused up to three times with the same RNA sample if larger numbers of transfected cells are required.
9. Thereafter, distribute the cells from the tube into independent wells containing an appropriate amount of prewarmed antibiotic free DMEM containing 10% FCS. As a general guidance, 150 µl of the cell suspension generated during step 1.2.8 are sufficient for a single well of a 24-

dish plate containing 0.5 ml of prewarmed DMEM, whereas 300 µl are appropriate for a well of a 12-dish plate containing 1 ml of prewarmed DMEM.

10. Incubate the cells at 37 °C and 10% CO_2 for 24 to 72 hours. Then, release infectious virions from cells by one (or more) freeze and thaw cycles and determine virus titre in the sample using either plaque assay or TCID50. Note that lysates should be clarified by centrifugation for 1-2 minutes at maximum speed or by their filtering through a 0.22 µm pore filter prior to titration. Typically, MNV reaches titres of around 1 x 10^6 TCID50/ml at 24 hours post-transfection and up to 1 x 10^9 at 72 hours post-transfection.
11. The presence and stability of mutations introduced in pT7:MNV 3'Rz are typically determined by sequencing the rescued viruses after 2 to 5 additional passages in Raw264.7 cells.

1.3 Recovery by lipofection into BHK-21 cells:

A more direct and often more cost effective method for the recovery of infectious MNV from capped transcripts is via lipofection (*Lipofectamine 2000*, Invitrogen). Given that Raw264.7 cells are difficult to transfect using lipid based approaches we normally make use of other easy to transfect cell lines such as BHK-21 which is an immortalised line derived from baby hamster kidney fibroblasts. As a standard approach in our laboratory we use BSR-T7 cells, a derivative of the BHK-21 cell line, as whilst these cells are easy to transfect and support MNV replication, they lack a suitable receptor to allow several rounds of re-infection. As a result, the virus yield generated from this system is an indication of a single cycle of virus replication. This approach is of particular use when examining the effect of mutation on virus recovery as it allows multiple transfections to be performed at substantially reduced costs compared to Neon-mediated transfection and also does not require specialist equipment. It is worth noting that other readily available cell lines such as human embryonic kidney 293T cells also support the efficient recovery of MNV however transfection conditions should first be optimised to ensure efficient RNA delivery.

1. Trypsinise a monolayer of BHK-21 cells (or BSR-T7 cells), seed 7.5 x 10^5 cells into a 35 mm diameter dish in antibiotic-free growth media and incubate the cells at 37 °C with 10% CO_2 overnight. Double the amount of cells in each plate if the transfections are planned for the same day as the seeding, and allow cells to adhere to the plate for 2-3 hours at 37 °C with 10% CO_2. Note that other cells that are suitable to this approach include human 293T cells, human hepatocellular carcinoma Huh7 cells and African green monkey Cos7 cells.
2. Remove the media from the cells and replace with 3 ml of fresh media without antibiotics to ensure the maximum efficiency of transfection.
3. Prepare a mixture of 1-2 µg of capped MNV transcript into 100 µl of Opti-MEM (Invitrogen) and mix it with 4 µl of *Lipofectamine 2000* previously mixed in 100 µl of Opti-MEM. Mix the sample thoroughly by pipetting it up and down 15 times. Leave the mixture at room temperature for 20 minutes.
4. Add the transfection complexes containing capped MNV transcripts in a drop-wise fashion to the cell monolayer and gently shake the plate in perpendicular directions.
5. Incubate the cells at 37 °C and 10% CO_2 for 24 to 72 hours. Afterwards, release infectious virions from cells by freeze and thawing and determine virus titre by plaque assay or TCID50. Typical yields of around 1 x 10^6 TCID50/ml are reached.

2. Direct Recovery of Infectious MNV from cDNA in Cells Expressing T7 RNA Polymerase

This protocol is designed to allow the recovery of MNV in cells by the transcription of an infectious plasmid harbouring the full genomic cDNA sequence by a T7 polymerase expressed in the cells. Different cell lines can be used to recover infectious MNV by this approach although we typically obtain the highest yields with BHK-21 and BSR-T7 cells [15]. We typically use BSR-T7 cells since they grow faster than the parental BHK clone line. Cells are infected with fowlpox (FPV) encoding for T7 RNA polymerase (FPV-T7) [18] which functions as a helper virus to drive expression of the viral RNA and subsequent recovery of infectious virus (**Figure 4**). Although BSR-T7 cells constitutively express T7 RNA polymerase, this expression is not sufficient to rescue infectious MNV after transfection of pT7:MNV 3'Rz in the absence of helper FPV-T7. Whilst the typical yields from this system are at least 10-fold lower than those described above, this approach does provide a rapid method of screening mutants to allow the identification of debilitating mutations. Typically this method is used first to assess the viability of a cDNA construct. Should a construct either fail to produce infectious virus or appear to yield virus at lower levels than that of the wild type infectious clone, then the RNA based approach described above is undertaken.

1. Trypsinise a monolayer of BHK-21 cells (or BSR-T7 cells) and seed 7.5 x 10^5 cells into a 35 mm dish in antibiotic free growth media and incubate the cells at 37 °C and 10% CO_2 overnight. Add double the amount of cells in each plate if the transfections are planned for the same day as the seeding, and allow cells to adhere to the plate for 2-3 hours at 37 °C and 10% CO_2.
2. Remove cell culture media and add 700 µl of FPV-T7 to each well (**Figure 4**). A multiplicity of infection (MOI) of ~0.5 PFU per cell, based on titrations on primary chicken embryo fibroblasts, is generally used. However it is worth noting that new preparations of helper virus grown in primary fibroblasts are functionally titrated to determine the dose required for efficient virus recovery. Protocols for the propagation and titration of FPV-T7 have been previously described [18].
3. Incubate at 37 °C and 10% CO_2 for 1 hour to allow FPV-T7 to infect the cells. Then, add 2 ml of antibiotic free DMEM containing 10% FCS and incubate the cells for an additional hour at 37 °C and 10% CO_2 to allow T7 RNA polymerase expression.
4. To proceed with the transfection of the infectious plasmid, firstly remove the media from the infected cells, wash with 2 ml of media (10% FCS in antibiotic free DMEM), and finally cover the cell monolayer with 3 ml of media. Antibiotics should not be added to the media since they may interfere with the efficiency of *Lipofectamine 2000* (Invitrogen).
5. Prepare a mix of 1 µg of wild type MNV cDNA infectious plasmid (e.g. pT7:MNV 3'Rz) in 100 µl of Opti-MEM (Invitrogen) and mix it with 4 µl of *Lipofectamine 2000* (Invitrogen) previously mixed with 100 µl of Opti-MEM (Invitrogen). Mix the reaction thoroughly by pipetting it up and down 15 times and keep the mix at room temperature for 20 minutes.
6. The resulting transfection mix should be then added drop-wise to the cell monolayer and the plate should be gently shaken in perpendicular directions.
7. Incubate the FPV-T7 infected, MNV plasmid-transfected cells at 37 °C and 10% CO_2 for 24 to 72 hours. Cells transfected with infectious plasmid pT7:MNV 3'Rz normally render titres from 1 x 10^4 to 5 x 10^4 TCID50/ml.

3. Representative Results

Both reverse genetics approaches are highly efficient for the recovery of infectious MNV in cell culture as shown in **Figure 5**. Infectious MNV with titres exceeding 10^5 TCID50/ml are recovered at 24 hours after transfection of capped MNV RNA into Raw264.7 cells. Similarly, the transfection of infectious plasmid pT7:MNV 3'Rz into BSR-T7 cells previously infected with helper FPV expressing T7 (FPV-T7) led to viral titres largely exceeding 10^4 TCID50/ml (**Figure 5**). These viral titre values obtained with synthetic RNA and DNA molecules are similar to those obtained in transfections involving natural VPg-linked RNA isolated from infectious virions into the same cells (**Figure 5**). These results highlight the high efficiency of the reverse genetics approaches described here to recover genetically defined MNV variants in cell culture.

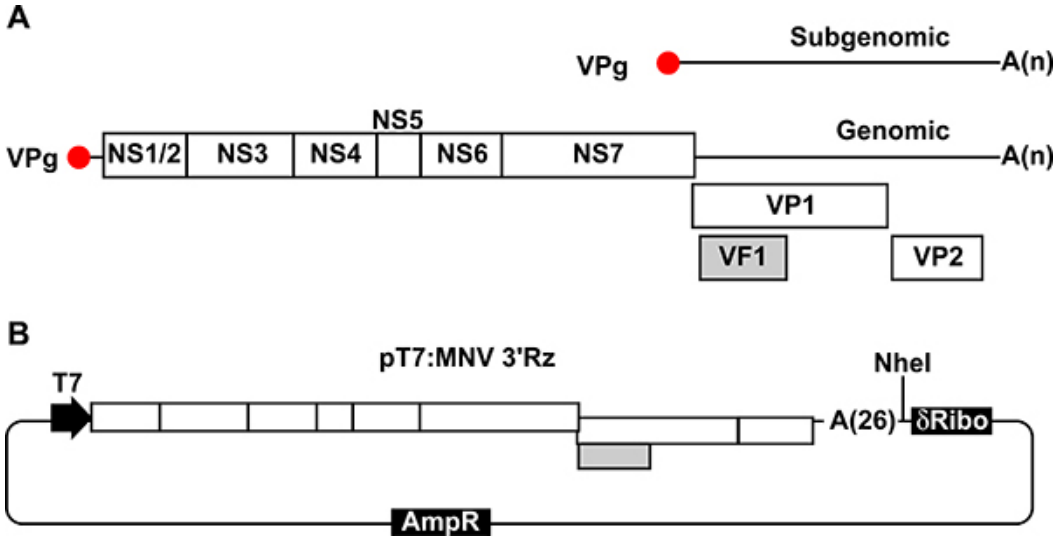

Figure 1. Illustration of MNV genome and plasmid for the recovery of infectious virus. A, Schematic representation of MNV genome organisation. Each protein coding region is illustrated as a single white box. ORF1 is translated into 7 different non-structural proteins (NS1/2 to NS7) that are released from precursor polyprotein after self-proteolytic processing. ORF 2 encodes the major capsid protein VP1, ORF 3 encodes the minor capsid protein VP2, and ORF4 overlapping with ORF2 coding region encodes virulence factor VF1. Genomic and subgenomic RNAs contain a polyA tail at their 3' ends of variable length. B, Plasmid containing MNV cDNA used in our reverse genetic approaches (pT7:MNV 3'Rz). MNV cDNA is fused to a polyA tail of 26 residues in its 3' end. The MNV cDNA sequence is located immediately downstream of a truncated T7 promoter sequence, to allow T7-driven transcription, and upstream of a unique *Nhe*I site and a DNA sequence coding for a self-cleaving ribozyme after it. These sequences are instrumental for ensuring RNA transcription termination right after the genomic polyA tail present at the 3' end.

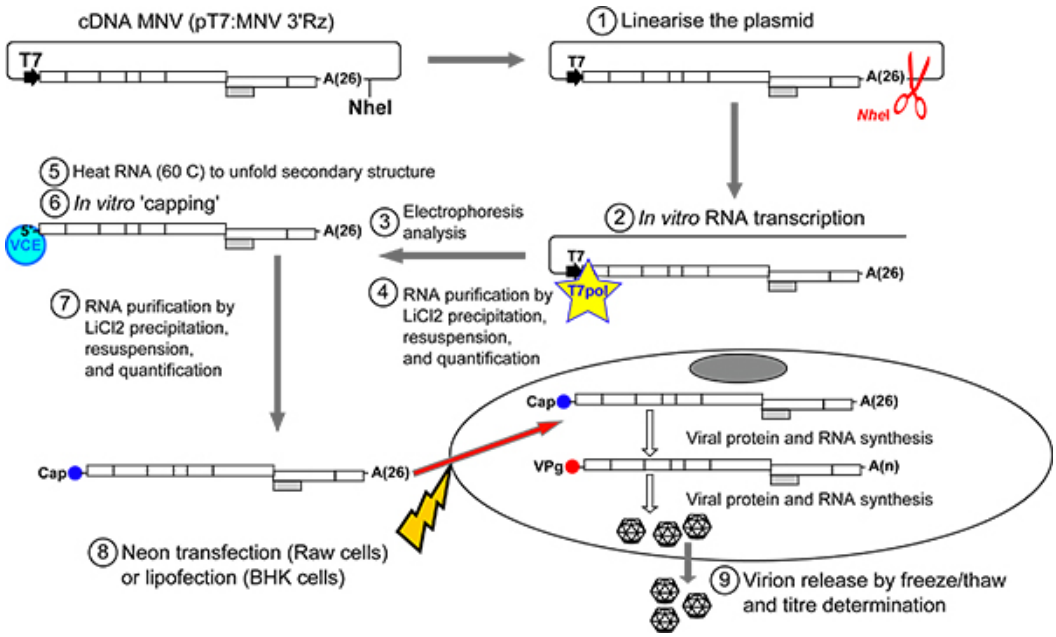

Figure 2. Overview of the protocol for the recovery of infectious MNV from RNA transcribed and capped *in vitro*. The plasmid pT7:MNV 3'Rz is linearised immediately downstream of the MNV genomic sequence using *Nhe*I restriction enzyme (step 1). After DNA purification, MNV RNA transcripts are generated *in vitro* by using T7 RNA polymerase (step 2). Transcription products usually run with an apparent mobility of 2.5-3Kb on a non-denaturing 1% agarose gel (step 3, **Figure 3**). The template DNA is eliminated using a commercial RNAse-free DNAse. RNA

is then purified from free nucleotides by LiCl precipitation (step 4). The purified RNA product may then be *in vitro* capped after being previously heated at 65 °C to unfold secondary RNA structures (steps 5-6). After purification by LiCl precipitation, RNA is transfected into either Raw264.7 cells (*Neon transfection system*, Invitrogen) or BSR-T7 cells (*Lipofectamine 2000*, Invitrogen) (steps 7-8). Once inside the cell, capped RNA transcripts will be translated into viral proteins which would catalyse viral transcripts replication into new MNV RNA molecules containing a proper VPg molecule at their 5' end. Successive cycles of replication accompanied of viral translation would generate large numbers of viral genomes which will be encapsidated to generate infectious virions. To facilitate virus release from cells, one or several cycles of freeze and thaw are performed (step 9). Viral yields can be then determined by TCID50 or plaque assay procedures.

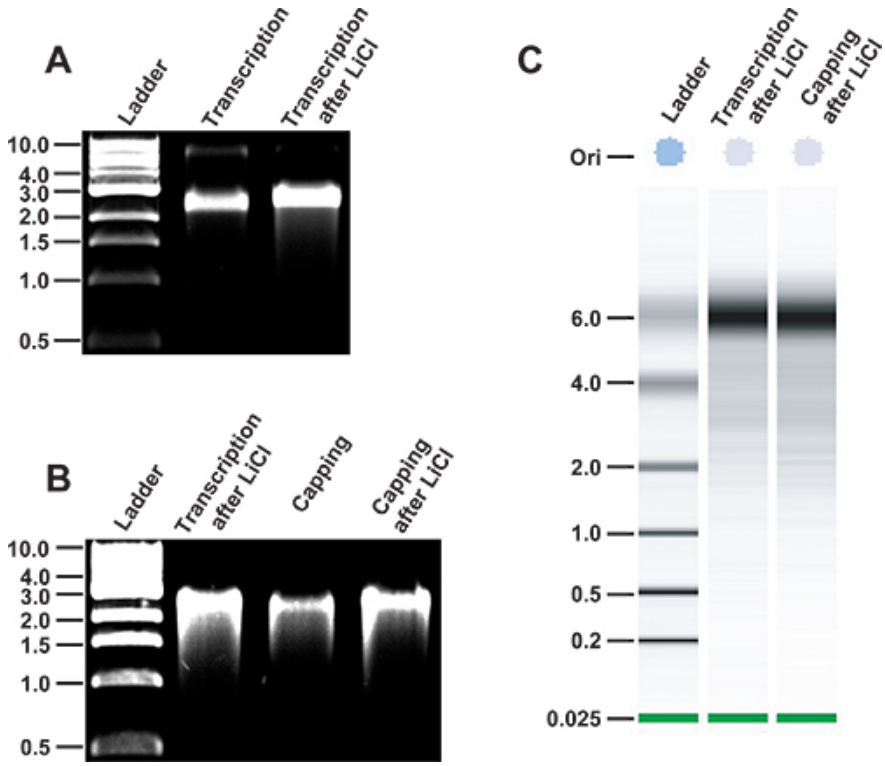

Figure 3. Analysis of MNV RNA transcripts integrity along the protocol. A, Integrity of MNV RNA synthesised *in vitro*. The plasmid pT7:MNV 3'Rz is firstly linearised using *Nhe*I restriction enzyme. After DNA purification, MNV RNA transcripts are generated *in vitro* by using T7 RNA polymerase (lane 2). RNA is then purified from free nucleotides by LiCl precipitation (lane 3). Transcription products are run on a non-denaturing 1% agarose gel in parallel to 1-Kb DNA ladder (New England Biolabs, lane 1). Relative mobility of viral transcripts under non-denaturing conditions is similar to a dsDNA product of 2.5-3 Kb. B, Integrity of MNV RNA transcripts after capping. MNV transcripts purified previously by LiCl precipitation (lane 2) are subjected to enzymatic capping (lane 3) and purification by LiCl precipitation (lane 4). C, , Analysis in an *Agilent RNA 6000 Nano chip* of MNV transcripts (second lane) and capped MNV transcripts (third lane) which have been previously precipitated in LiCl. A ssRNA ladder is run in parallel.

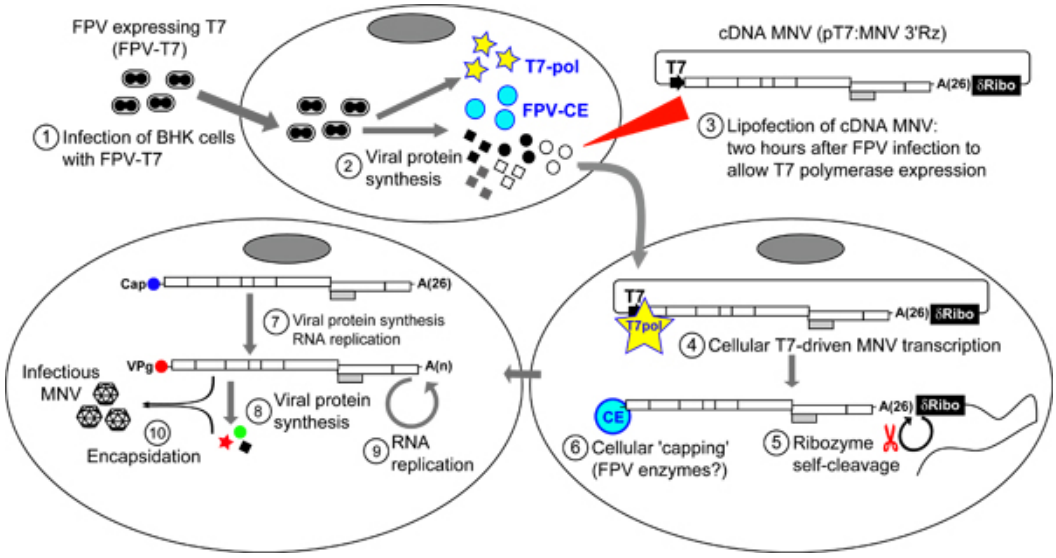

Figure 4. Overview of the protocol for the recovery of infectious MNV from cDNA. Initially, BSR-T7 (or BHK) cells are infected with a recombinant fowlpox virus (FPV) expressing the bacteriophage T7 RNA polymerase (FPV-T7) (step 1). The infected cells are incubated for 2 hours before further treatment to allow the expression of FPV proteins which includes the recombinant T7 RNA polymerase (step 2). Afterwards, pT7:MNV 3'Rz is transfected into the cells by using *Lipofectamine 2000* (Invitrogen) (step 3). Once inside the cell, pT7:MNV 3'Rz is recognised by T7 RNA polymerase which synthesises MNV RNA transcripts (step 4). The presence of a self-cleaving δ-Ribozyme sequence at the 3'end of the genome guarantees the transcript 3' terminus is located just after the polyA tail (step 5). Some viral transcripts are intracellularly capped by an FPV capping enzyme (step 6). The resulting MNV capped transcripts will be translated to generate MNV proteins which would catalyse MNV transcripts replication. Newly synthesised MNV RNA molecules containing a proper VPg molecule at their 5' end would undergo successive cycles of replication accompanied by viral translation which may finally result in the generation of infectious encapsidated virus. To facilitate virus release from cells, one or several cycles of freeze and thaw are performed (step 7). Viral yields can be then determined by TCID50 or plaque assay procedures.

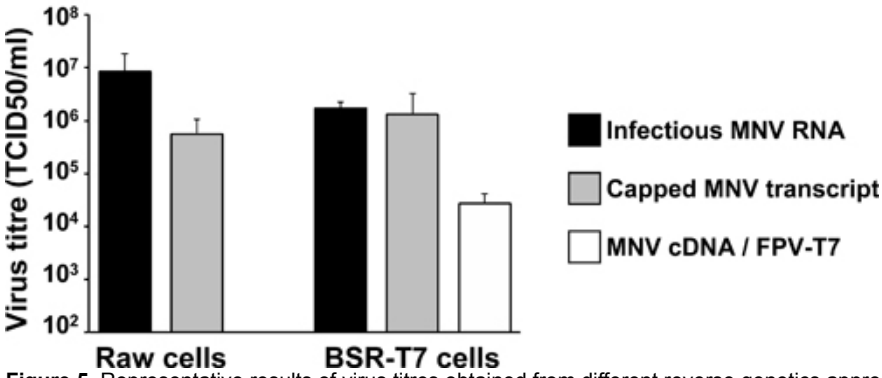

Figure 5. Representative results of virus titres obtained from different reverse genetics approaches described in the text. Grey bars represent the virus titres obtained at 24 hours after Neon-transfection of 2 x 10^6 Raw264.7 cells, or after lipo-transfection of 2 x 10^6 BSR-T7 cells with *in vitro* transcribed and capped MNV RNA. White bars represent the virus titre typically obtained after lipofection of pT7:MNV 3'Rz (MNV cDNA) into 2 x 10^6 BSR-T7 cells previously infected for 2 hours with fowlpox virus expressing recombinant T7 polymerase (FPV-T7). As a positive control for the transfection into Raw and BSR-T7cells, we typically use 2 µg of RNA extracted from cells infected with MNV which contain high levels of VPg-linked MNV RNA. Negative controls have been carried out with either MNV RNA or pT7:MNV 3'Rz encoding a frameshift mutation (F/S) which abrogates replication, resulting in no detectable virus (data not shown).

Discussion

Here we have illustrated two different reverse genetic approaches that allow the recovery of infectious MNV in cell culture. Both approaches effectively bypass the absolute requirement for the covalently linkage of VPg to the 5' end of the viral RNA genome via the generation of capped MNV transcripts that are then recognised by the cellular ribosomes. *In vitro* transcription followed by enzymatically capping is more efficient in the recovery of infectious MNV than transcription of infectious plasmids in cells expressing T7 RNA polymerase, in which the transcripts may be capped by the FPV capping enzymes. Virus titres recovered with these reverse genetics systems are similar to these obtained by transfection of viral VPg-linked RNA purified from infected cell cultures [17] (**Figure 5**). The transfection of capped MNV RNA into permissive Raw264.7 cells renders a virus titre only 1-log lower than experiments involving the transfection of total RNA from infected cells containing viral VPg-linked RNA (**Figure 5**). This fact encourages further investigations to determine whether the addition of a VPg molecule to the 5' end of transcripts generated by these systems could result in increased virus yields which may reveal functional aspects underlying MNV infectivity in cells associated to

VPg. Nevertheless, we regard this reverse genetics system as a highly efficient one comparable to other RNA viruses reverse genetics systems currently used in which the *in vitro* transcribed RNA allows the recovery of titres only 10-100 lower than in real infections with virions [19, 20].

Overall, the current methodologies constitute a significant step forward in the field of norovirus molecular biology and provide us with the tools to investigate the functional roles of proteins and conserved RNA motifs in norovirus genomes. These approaches have already been combined with current mouse model available and have shown that MNV recovered from infectious cDNA is able to cause lethal infection of >80% STAT1-/- mice in less than 10 days [4, 21]. Making use of this system we have recovered viable murine norovirus mutants of the capsid protein and of a polypyrimidine tract involved in the binding of different host factors (PTB and PCBP) that display a somewhat attenuated phenotypes *in vivo* [21, 22]. In addition, we have recently demonstrated that viruses lacking the ability to express the VF1 protein from ORF4 efficiently replicate in cell culture but again have reduced virulence in mice with respect to WT MNV [8]. These studies encourage us to design attenuated versions of human noroviruses based on MNV studies which could be investigated as potential vaccine candidates.

Disclosures

We have nothing to disclose.

Acknowledgements

This research was funded by a Wellcome Trust Senior Fellowship awarded to Ian Goodfellow, and a Marie Curie Intra European Fellowship (FP7 European Research Council) awarded to Armando Arias. We would like to thank Io Hong Cheong, Dr Rebecca Robey and Dr Mike Skinner for giving us permission to use their Agilent bioanalyser and helping with running RNA samples.

References

1. Outbreaks of gastroenteritis associated with noroviruses on cruise ships--United States, 2002. *MMWR Morb. Mortal. Wkly. Rep.* **51**, 1112-5 (2002).
2. Lopman, B.A., *et al*. Epidemiology and cost of nosocomial gastroenteritis, Avon, England, 2002-2003. *Emerg. Infect. Dis.* **10**, 1827-34 (2004).
3. Duizer, E. *et al*. Laboratory efforts to cultivate noroviruses. *J. Gen. Virol.* **85**, 79-87 (2004).
4. Karst, S.M., Wobus, C.E., Lay, M., Davidson, J., & Virgin, H.W.T. STAT1-dependent innate immunity to a Norwalk-like virus. *Science*. **299**, 1575-8 (2003).
5. Wobus, C.E., *et al*. Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. *PLoS Biol.* **2**, e432 (2004).
6. Bok, K., *et al*. Inhibition of norovirus replication by morpholino oligomers targeting the 5'-end of the genome. *Virology*. **380**, 328-37 (2008).
7. Kim, Y., Thapa, M., Hua, D.H., & Chang, K.O. Biodegradable nanogels for oral delivery of interferon for norovirus infection. *Antiviral Res*. **89**, 165-73 (2011).
8. McFadden, N., *et al*. Norovirus Regulation of the Innate Immune Response and Apoptosis Occurs via the Product of the Alternative Open Reading Frame 4. *PLoS Pathog*. **7**, e1002413 (2011).
9. Kormelink, R., van Poelwijk, F., Peters, D., & Goldbach, R. Non-viral heterogeneous sequences at the 5' ends of tomato spotted wilt virus mRNAs. *J. Gen. Virol.* **73** (Pt. 8), 2125-8 (1992).
10. Shuman, S. & Hurwitz, J. Mechanism of mRNA capping by vaccinia virus guanylyltransferase: characterization of an enzyme--guanylate intermediate. *Proc. Natl. Acad. Sci. U.S.A*. **78**, 187-91 (1981).
11. Jang, S.K., Pestova, T.V., Hellen, C.U., Witherell, G.W., & Wimmer, E. Cap-independent translation of picornavirus RNAs: structure and function of the internal ribosomal entry site. *Enzyme*. **44**, 292-309 (1990).
12. Chaudhry, Y., *et al*. Caliciviruses differ in their functional requirements for eIF4F components. *J. Biol. Chem*. **281**, 25315-25 (2006).
13. Goodfellow, I., *et al*. Calicivirus translation initiation requires an interaction between VPg and eIF 4 E. *EMBO Rep*. **6**, 968-72 (2005).
14. Herbert, T.P., Brierley, I., & Brown, T. D. Identification of a protein linked to the genomic and subgenomic mRNAs of feline calicivirus and its role in translation. *J. Gen. Virol.* **78** (Pt 5), 1033-40 (1997).
15. Chaudhry, Y., Skinner, M.A., & Goodfellow, I.G. Recovery of genetically defined murine norovirus in tissue culture by using a fowlpox virus expressing T7 RNA polymerase. *J. Gen. Virol.* **88**, 2091-100 (2007).
16. Ward, V.K., *et al*. Recovery of infectious murine norovirus using pol II-driven expression of full-length cDNA. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 11050-5 (2007).
17. Yunus, M.A., Chung, L.M., Chaudhry, Y., Bailey, D., & Goodfellow, I. Development of an optimized RNA-based murine norovirus reverse genetics system. *J. Virol. Methods.* **169**, 112-8 (2010).
18. Arias, A., Perales, C., Escarmis, C., & Domingo, E. Deletion mutants of VPg reveal new cytopathology determinants in a picornavirus. *PLoS One*. **5**, e10735 (2010).
19. van der Werf, S., Bradley, J., Wimmer, E., Studier, F.W. & Dunn, J.J. Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2330-4 (1986).
20. Bailey, D., Thackray, L.B., & Goodfellow, I.G. A single amino acid substitution in the murine norovirus capsid protein is sufficient for attenuation *in vivo*. *J. Virol.* **82**, 7725-8 (2008).
21. Bailey, D. *et al*. Functional analysis of RNA structures present at the 3' extremity of the murine norovirus genome: the variable polypyrimidine tract plays a role in viral virulence. *J. Virol.* **84**, 2859-70 (2010).