Modeling The Lifecycle Of Ebola Virus Under Biosafety Level 2 Conditions With Virus-like Particles Containing Tetracistronic Minigenomes

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Abstract

Ebola viruses cause severe hemorrhagic fevers in humans and non-human primates, with case fatality rates as high as 90%. There are no approved vaccines or specific treatments for the disease caused by these viruses, and work with infectious Ebola viruses is restricted to biosafety level 4 laboratories, significantly limiting the research on these viruses. Lifecycle modeling systems model the virus lifecycle under biosafety level 2 conditions; however, until recently such systems have been limited to either individual aspects of the virus lifecycle, or a single infectious cycle. Tetracistronic minigenomes, which consist of Ebola virus non-coding regions, a reporter gene, and three Ebola virus genes involved in morphogenesis, budding, and entry (VP40, GP₁₂, and VP24), can be used to produce replication and transcription-competent virus-like particles (trVLPs) containing these minigenomes. These trVLPs can continuously infect cells expressing the Ebola virus proteins responsible for genome replication and transcription, allowing us to safely model multiple infectious cycles under biosafety level 2 conditions. Importantly, the viral components of these systems are solely derived from Ebola virus and not from other viruses (as is, for example, the case in systems using pseudotyped viruses), and VP40, GP₁₂, and VP24 are not overexpressed in this system, making it ideally suited for studying morphogenesis, budding and entry, although other aspects of the virus lifecycle such as genome replication and transcription can also be modeled with this system. Therefore, the tetracistronic trVLP assay represents the most comprehensive lifecycle modeling system available for Ebola viruses, and has tremendous potential for use in investigating the biology of Ebola viruses in future. Here, we provide detailed information on the use of this system, as well as on expected results.

Video Link

The video component of this article can be found at https://www.jove.com/video/52381/

Introduction

Ebola viruses are the causative agent of a severe hemorrhagic fever in humans and non-human primates with case fatality rates of up to 90% in human outbreaks⁠. While there has been significant progress in recent years in developing vaccines as well as specific treatments (reviewed in⁠,⁠), these are not approved for human use. Ebola virus particles have a characteristic thread-like appearance with a length of about 1 µm and a diameter of 96 to 98 nm⁠. A nucleocapsid forms the core of the viral particles and consists of 1) the non-segmented single-stranded negative-sense RNA genome, which encodes the 7 ebolavirus genes (Figure 1, 2) the nucleoprotein NP, which encapsidates the genome, 3) the viral polymerase complex consisting of the polymerase L and its cofactor VP35, and 4) the transcriptional activator VP30. In addition, it has recently been shown that the protein VP24 is also associated with nucleocapsids⁠. The nucleocapsid is surrounded by the matrix space in which the matrix protein VP40, which is responsible for morphogenesis and budding of virions, is located. Virus particles are further enveloped, and embedded in that envelope is the Golgi membrane protein GP₁₂, which is responsible for virion attachment and entry.

Work with infectious Ebola viruses has to be performed in a maximum containment laboratory under biosafety level (BSL) 4 conditions, which restrict this work to a few facilities worldwide. In order to study the biology of these viruses or to develop novel therapeutics under BSL2 conditions, researchers rely either on recombinant overexpression of Ebola virus proteins, or on lifecycle modeling systems, both of which can be worked with in the absence of infectious Ebola virus. Recombinant expression of Ebola virus proteins is either achieved from expression plasmids or viral vectors. A special case of this strategy is the generation of virions or virus-like particles based on viruses other than Ebola viruses (most commonly retroviruses or vesicular stomatitis virus) in the presence of recombinantly expressed GP₁₂, leading to the generation of pseudotyped particles, which can be used to study the entry process of filoviruses and screen for entry inhibitors⁠. Alternatively, recombinant viruses (e.g., vesicular stomatitis virus) that encode the Ebola virus GP₁₂ instead of their own glycoprotein can be generated and used to study virus entry under biosafety level 2 conditions⁠.

Lifecycle modeling systems are forms of reverse genetics systems featuring the use of truncated Ebola virus genome analogues (minigenomes), which are initially produced from cDNA and then replicated and transcribed by Ebola virus proteins provided in trans. The first minigenome system for Ebola virus was developed more than 15 years ago⁠, and has since been used to study Ebola virus genome replication and...
transcription (reviewed in 8,9). In this system a monocistronic minigenome consisting of a single reporter gene flanked by the terminal non-coding regions of the Ebola virus genome (called the leader and trailer) (Figure 1) is expressed in mammalian cells (usually by transcription using T7 RNA polymerase) together with the viral proteins L, VP35, VP30 and NP. The minigenome is encapsidated by NP, and then replicated and transcribed by the other nucleocapsid proteins using cis-acting signals localized in the leader and trailer, leading to reporter activity that reflects these two aspects of the virus lifecycle (Figure 2).

In order to model additional steps of the viral lifecycle, transcription and replication-competent virus-like particle (trVLP) systems have been developed, which are based on classical minigenome systems, but feature the additional expression of the other viral proteins VP24, VP40 and GP, from expression plasmids10,11. The presence of VP40 leads to the formation of trVLPs, which bear GP on their surface, and carry a minigenome-containing nucleocapsid on the inside. These trVLPs can be used to infect target cells, which have either been pretransfected with expression plasmids for L, VP35, VP30 and NP, to facilitate replication and transcription of minigenomes brought into the target cells within trVLPs11, or are naive target cells (i.e. without plasmid-driven expression of Ebola virus proteins)10. This results in reporter activity in target cells, which reflects the replication of the minigenomes in the producer cells, morphogenesis and budding of trVLPs, their entry into target cells, and 1) in the case of pretransfected target cells also genome replication and secondary transcription (i.e. transcription by viral proteins produced in target cells) in target cells, or 2) in the case of naive target cells also primary transcription (i.e. transcription by viral proteins brought into target cells within trVLPs) (Figure 3). Importantly, these systems have only been used to model a single infectious cycle, and rely on overexpression of all viral proteins, which in the case of VP24 and VP40 is particularly problematic, since these proteins have been shown to be strong negative regulators of genome replication and transcription when overexpressed from plasmids12,13. Further, trVLP preparations produced in these systems contain a high proportion of non-infectious particles, posing challenges for the biochemical analysis of infectious trVLPs14.

In order to overcome these problems, we have recently developed a tetracistronic minigenome system that, in addition to a reporter gene, also contains the genes encoding for VP40, GP, and VP24 (Figure 1). Similar to the classical monocistronic minigenome system, this system leads to the production of trVLPs that can infect target cells (Figure 4)15-17. However, in contrast to the classical minigenome system, VP40, GP, and VP24 are produced after viral genome transcription rather than being overexpressed from plasmids. As a result, the kinetics and expression levels of these proteins much more closely mimic those found during the viral lifecycle, and consequently the ratio of infectious to non-infectious trVLPs is increased about 500-fold in this system15. Further, using this system it was possible to continuously passage tetracistronic minigenome-containing trVLPs, modeling multiple infectious cycles. As such, tetracistronic trVLPs are currently the most comprehensive lifecycle modeling system available to study Ebola virus biology under BSL2 conditions. Here, we provide detailed information on the use of this system, as well as on expected results.

### Protocol

#### 1. Splitting of Producer Cells for Initial Production of trVLPs

1. Remove medium from 80-90% confluent 293 cells cultured in 75 cm² flasks in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1x pen/strep (DMEM10). Wash cells twice with 10 ml phosphate-buffered saline (PBS), being careful not to dislodge the cells, and add 2 ml trypsin-EDTA to the cells.
2. Incubate the cells at room temperature until cells show significant rounding when observed under a microscope (about 30 sec). Dislodge cells by tapping flask, and add 8 ml DMEM10. Thoroughly resuspend the cells by gently pipetting up and down until a single cell suspension is observed when viewed under the microscope.
3. Count the cells using an automated cell counter. Dilute cells to 2 x 10⁵ cells per ml in DMEM10. Pipette 2 ml of cell suspension per well into 6-well plates (4 x 10⁴ cells per well).
4. Incubate the plates in a humidified tissue culture incubator at 37 °C with 5% CO₂.

#### 2. Transfection of Producer Cells for Initial Production of trVLPs

1. 24 hr after splitting the cells (see Figure 5 for an overview of the experiment timing), pipette plasmid DNA15 (for amounts see Table 1) into a sterile 2 ml cryovial using filtered tips. Add 100 µl OptiMEM per well to the DNA. Vortex the mixture briefly and gently spin down the tubes using a microfuge. If several wells are to be transfected with identical plasmids, a mastermix for several wells can be made.
2. Briefly vortex the vial with Transit LT1 before use. Add 7.5 µl Transit LT1 per well to the diluted DNA. Gently vortex the mixture, taking care not to collect liquid in the lid of the cryovial, and incubate for 15 min at room temperature.
3. Gently mix the transfection complex by pipetting up and down. Add 100 µl of the transfection complexes dropwise to each well. Rock the plate forward/backward and from side to side to distribute the transfection complexes. Do not swirl the plate as this will cause uneven spreading of the transfection complexes.
4. Return the cells to the incubator.
5. After 24 hr, remove the supernatant from cells. Add 4 ml DMEM with 5% FBS, 2 mM L-glutamine, 1x pen/strep (DMEM5) to the cells. This step can be done for up to 3 wells at a time without drying of the wells, assuming the wells contain identical samples (otherwise, due to the self-amplifying nature of the trVLPs in this system, cross-contamination can become an issue and this step should be done one well at a time).
6. Return the cells to the incubator.

#### 3. Preparation of Target Cells

1. Split 293 cells as described in section 1, seeding 4 x 10⁵ cells in 2 ml DMEM10 per well of a 6-well plate.
2. 24 hr after splitting of the target cells and 24 hr before infection, transfect target cells as described in section 2 using the DNA amounts in Table 1, and 4.5 µl Transit LT1 per well.
4. Infection of Target Cells and Harvest of Producer Cells

1. Transfer the supernatants from the producer cells to 15 ml tubes. Remove and discard any remaining supernatant using a vacuum hose. Add 250 μl Glo lysis buffer to the cells.
2. Centrifuge supernatant for 5 min at 800 x g and room temperature to clear the samples of cellular debris.
3. Remove the supernatant from one well of the target cells. Carefully add 3 ml of cleared producer cell supernatant to target cells using the slowest pipette speed. Avoid pipetting directly onto the cells in order to avoid disrupting the cell monolayer. This step has to be done one well at a time.
4. When all samples have been transferred, return the target cells to the incubator to allow sedimentation of trVLPs and infection of target cells.
5. After 15 min of incubation at room temperature in the Glo lysis buffer, resuspend the producer cells in the lysis buffer using a micropipette set to 150 μl, and transfer the sample into a 2 ml cryovial. At this point, lysates can be frozen at -80 °C, or directly measured as described in section 6.
6. 24 hr after infection, remove the supernatant from the target cells. Add 4 ml DMEM5 per well to the cells. This step can be done for up to three wells at a time, if the wells contain identical samples (otherwise, due to the self-amplifying nature of the trVLPs in this system, cross-contamination can become an issue, and this step should be done one well at a time).

5. Harvest of Target Cells for a Single Cycle Infection

1. If only a single infectious cycle should be assessed, at 72 hr after infection remove the supernatant from the target cells using a vacuum hose.
2. Harvest the cells in 250 μl Glo lysis buffer as described in section 4 for producer cells.

6. Harvest of Target Cells and Continuous Passaging of trVLPs

1. If trVLPs are to be continuously passaged, prepare a new set of target cells as described in section 3 so that they are ready for infection 72 hr after infection of the first set of target cells (see Figure 5).
2. Infect the new set of target cells as described in section 4 (using the first set of target cells in place of the producer cells).
3. Repeat these steps every 72 hr.

7. Analysis of Reporter Activity

1. If cell lysates were frozen, thaw them at room temperature. Ensure that samples have reached room temperature prior to measurement.
2. Thaw the required amount (40 µl per sample) of Renilla Glo assay buffer (ideally frozen in aliquots) at room temperature. Ensure that the buffer has reached room temperature prior to measurement.
3. Add 1/1000 volume of Renilla Glo substrate to the Renilla Glo assay buffer to obtain Renilla Glo reagent, and mix by vortexing. Pipette 40 µl of the Renilla Glo reagent into a white 96-well plate.
4. Add 40 µl of sample to the Renilla-Glo reagent. Wait 10 min, then measure the samples in a luminometer using an integration time of 1 sec.

Representative Results

Transfection of 293 producer cells with expression plasmids encoding the Ebola virus nucleocapsid proteins NP, VP35, VP30, and L, a tetracistronic minigenome and the accessory T7 polymerase results in minigenome replication and transcription and reporter activity that is readily detectable at 72 hr (Figure 6). Importantly, the observed reporter activity (10⁻⁶⁵ relative luminescence units (RLU)) exceeds that of a negative control in which the expression plasmid encoding L was omitted from the transfection (10⁻⁵ RLU) by more than 2 logs. The low level of activity observed even in absence of L is most likely due to a cryptic promoter in the minigenome plasmid. Target cells infected with trVLPs from the supernatant of producer cells actually show somewhat increased levels of reporter activity when compared to producer cells, and values reach between 10⁷ and 10⁸ RLU, depending on the passage. In contrast, when the supernatant of –L control producer cells is passaged onto target cells (expressing all nucleocapsid protein, including L), reporter activity does not exceed the background noise of the luminometer (about 10⁵ RLU).

The tetracistronic trVLP assay can be used to study the lifecycle of Ebola viruses. For example, infection of 293 cells with trVLPs is dependent on the presence of attachment factors such as Tim-1 which has to be provided in trans in these cells. Consequently, in a tetracistronic trVLP assay a drop in reporter activity in target cells of about 100-fold is observed in the absence of Tim-1 (Figure 7A). The role of specific (viral or cellular) proteins in the virus lifecycle can also be assessed using RNAi technology together with this approach. As an example, RNAi-mediated down-regulation of L results in a drop in reporter activity of about 100-fold, reflecting the central role of L in replication and transcription (Figure 7B). Further, it is possible to directly manipulate the minigenome to assess the effect of mutations on the virus lifecycle. As an example, when VP24-expression from the minigenome is abolished by introducing 3 stop codons immediately downstream of the start codon, reporter activity in producer cells is not dramatically changed; however, reporter activity in target cells is reduced by about 20-fold after one passage, and 400-fold after two passages, indicating a role of VP24 in the production of infectious trVLPs (Figure 7C).
Figure 1. Structure of the Ebola virus genome as well as monocistronic and tetracistronic minigenomes. Coding regions for the Ebola virus protein are shown as red (NP, VP35, VP30, and L), yellow (VP40 and VP24) or blue (GP) boxes. Non-coding regions (NCRs) are shown in green, with the leader and trailer regions indicated. The subscript indicates which viral NCR was used for joining the coding regions. The coding region for the reporter (rep) is shown in purple.

Figure 2. Monocistronic minigenome assay. Cells are transfected with expression plasmids for the Ebola virus nucleocapsid proteins (NP, VP35, VP30, L), a monocistronic minigenome (mg) and the T7 polymerase. The minigenome is initially transcribed by the T7 polymerase (a) into a unencapsidated minigenome RNA in the same orientation as the viral genome (vRNA), which is then encapsidated by NP (b). This encapsidated vRNA is replicated via a complementary minigenome RNA (cRNA) intermediate (c), and then transcribed into reporter mRNAs (d) that are translated into reporter protein (e).
Figure 3. trVLP assay with a monocistronic minigenome. Cells are transfected with expression plasmids for the minigenome assay components (the Ebola virus nucleocapsid proteins NP, VP35, VP30, L, a monocistronic minigenome and the T7 polymerase) as well as VP40, GP₁,₂ and VP24. This leads to the formation of trVLPs that incorporate minigenome-containing nucleocapsids (f). These trVLPs can then infect target cells (g), which are either pretransfected with expression plasmids for NP, VP35, VP30, and L (top), resulting in replication and secondary transcription (d) leading to reporter expression (e), or naive target cells (bottom), resulting in primary transcription of the minigenomes (h), also leading to reporter expression (e).
Figure 4. trVLP assay with a tetracistronic minigenome. Cells are transfected with expression plasmids for the Ebola virus nucleocapsid proteins (NP, VP35, VP30, L), a tetracistronic minigenome (mg) and the T7 polymerase. Initial transcription (a), encapsidation (b), genome replication (c) and transcription (d) as well as translation (e) occur as in a monocistronic minigenome assay. However, in addition to reporter mRNA, mRNAs for VP40, GP$_{1,2}$ and VP24 are also transcribed from the tetracistronic minigenome, resulting in the formation of trVLPs (f). These trVLPs infect target cells that have been pretransfected with expression plasmids for the nucleocapsid proteins NP, VP35, VP30 and L, as well as the cellular Ebola virus attachment factor Tim-1, resulting in genome replication and transcription, and production of trVLPs that can be used to infect fresh target cells.

Figure 5. Timing of a tetracistronic trVLP assay for 3 consecutive passages. The days for seeding cells (s), transfecting cells (t), infecting cells (i), medium change (c) and harvesting of cells and trVLPs (h) are indicated for three consecutive passages (indicated by arrows).
Figure 6. Typical levels of reporter activity observed in a tetracistronic trVLP assay. A tetracistronic trVLP assay was performed over 5 passages following the protocol outlined in this manuscript. As a negative control the expression plasmid encoding L was omitted (-L) from transfection of the p0 producer cells. Target cells in passages p1 to p5 were transfected with expression plasmids for all nucleocapsid proteins, including L. The background noise of the luminometer is indicated as a dashed line. Means and standard deviations of 4 biological replicates from 3 independent experiments are shown.
Figure 7. Assessing the impact of various viral and cellular factors on the viral lifecycle using tetracistronic trVLPs. (A) Tim-1 as an attachment factor. Target cells that were pretransfected with expression plasmids encoding for the nucleocapsid proteins and with or without a plasmid encoding for Tim-1 (described in \(^{15}\)) were infected with tetracistronic trVLPs. 72 hr post infection reporter activity in p1 target cells was determined. Means and standard deviations of 4 biological replicates from 4 independent experiments are shown. (B) Effect of RNAi knockdown of L on genome replication and transcription. Target cells that were pretransfected with expression plasmids for the nucleocapsid components, Tim-1 and miRNAs directed against L (anti-L) or an unrelated protein (anti-GFP) (described in \(^{15}\)) were infected with tetracistronic trVLPs. 72 hr post infection reporter activity in p1 target cells was determined. Means and standard deviations of 5 biological replicates from 2 independent experiments are shown. (C) Effect of mutations in the minigenome on trVLP infectivity. A tetracistronic trVLP assay was performed using either a wild-type minigenome (4cis-WT) or a minigenome in which 3 stop codons had been introduced immediately after the start codon of VP24, abolishing expressing of this protein but introducing only minimal changes to the minigenome in regards to length, nucleic acid composition, secondary structures etc. Reporter activity in p0, p1 and p2 was measured 72 hr after transfection / infection. Means and standard deviations of 3 biological replicates from 3 independent experiments are shown.

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<th>Producer Cells (p0)</th>
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Discussion

The tetracistronic trVLP assay described in this manuscript allows modeling of the Ebola virus lifecycle over several infectious cycles. Importantly, the trVLPs produced in this system do not contain the genetic information for the nucleocapsid proteins NP, VP35, VP30, and L, which together make up almost 60% of the Ebola virus genome and are essential for viral replication. Rather, these proteins have to be provided in target cells in trans from expression plasmids, and any infection of cells not expressing all 4 of those proteins is abortive. Importantly, there is no evidence of genetic recombination for flaviviruses, and there are no homologous regions shared between the tetracistronic minigenome and the expression plasmids for the nucleocapsid proteins. Therefore, there is neither any practical evidence nor any theoretical basis that could provide for the possibility of generating full length Ebola virus genomes that could then potentially result in the production of infectious Ebola viruses, making this system safe for use under BSL2 conditions.

There are two critical steps in the tetracistronic trVLP assay that are influenced by experimental conditions, i.e. the production of trVLPs, and the infection of target cells with these trVLPs. Production of trVLPs is dependent on high levels of minigenome replication and transcription, which in turn is dependent on a high transfection efficacy. Transfection efficacy can be easily assessed by including a -L control, in which the expression plasmid for L is exchanged for an expression plasmid encoding eGFP. Transfection rates under these conditions should exceed 50% by inspection with a fluorescence microscope 24 hours post transfection. Further, cells have to be free of mycoplasma, since in our experience mycoplasma contamination dramatically impairs minigenome replication and transcription (unpublished data). Due to their high transfectability 293 cells are the cell line of choice for trVLP assays; however, these cells are relatively poorly susceptible (albeit not completely refractive) to infection with Ebola virus. Expression of an attachment factor such as Tim-1 enhances infection of 293 cells with trVLPs about 100-fold, and is crucial for the success of this system over several passages.

While tetracistronic trVLPs are not self-replicating on their own, they are self-replicating in cells that express the nucleocapsid proteins. As such, precautions have to be made to avoid cross-contaminations between wells containing different trVLPs (e.g., with different mutations in the minigenome). From a more technical standpoint, due to the large difference between positive and negative signals in this assay (about 4 logs), cross-talk between samples when measuring luciferase activity can be an issue; however, this can be easily avoided by leaving one empty well between each sample in the 96-well plate used for measuring luciferase activity.

There are a multitude of possible applications for tetracistronic trVLPs. Obviously, they are well suited to studying the entry of flavivirus particles, since infectious trVLPs have the typical thread-like structure of infectious flaviviruses, and contain the same viral components as flavivirus particles. Importantly, they do not contain components of other viruses, as is the case when using pseudotyped virions or virus-like particles, such as retrovirus particles bearing GP1,2, or recombinant viruses, such as recombinant vesicular stomatitis virus expressing GP1,2. The requirement for attachment factors when using 293 cells as target cells in this system can be exploited to screen for and investigate the role of such attachment factors, while the roles of other cellular factors, such as those involved in genome replication and transcription as well as morphogenesis and budding, can be investigated using RNAi technology. The effect of mutations in viral proteins on the virus lifecycle can also be studied, although one has to keep in mind that while VP40, GP1,2, and VP24 are expressed after viral transcription, expression of the other viral proteins is achieved from expression plasmids, so that effects due to the overexpression of these proteins have to be taken into account. Also, care should be taken that mutations in the minigenome do not significantly alter the length of the minigenome, since reporter activity is directly affected by minigenome length. Finally, since tetracistronic minigenomes are viral genome analogues that carry viral genes, and are replicated by the viral polymerase complex, it should also be possible to study the evolution of these genes in response to mutations under BSL2 conditions. Thus, while further studies are still needed in this direction, it should be possible, for example, to introduce suboptimal mutations in genes within the minigenome and then passage trVLPs until complimentary mutations emerge.

One limitation of the tetracistronic trVLP assay that has to be kept in mind is the fact that while it models most of the virus lifecycle, primary transcription in target cells is not modeled by this system, since target cells have to express the nucleocapsid proteins in trans in order for the trVLPs to replicate. If primary transcription has to be assessed, it is possible to use naive target cells; however, in this case no genome replication takes place in target cells, and no further infectious trVLPs are produced, aborting the infection. This is a principal problem that cannot be overcome without rendering the trVLPs completely self-replicating, by including the genes for the nucleocapsid proteins into the minigenome, which would de facto turn them into infectious recombinant Ebola viruses. In fact, Ebola viruses expressing luciferase or other reporters have been generated and can be utilized to assess and study genome replication and transcription; however, their use is restricted to BSL4 laboratories. Also, it has to be kept in mind that while VP40, GP1,2, and VP24 are expressed from a viral genome analogue, their position in the minigenome (2nd, 3rd, and 4th transcriptional unit) is not identical to their position in the viral genome (3rd, 4th, and 5th transcriptional unit), which could influence their absolute expression levels, as well as their relative expression levels with respect to one another.

Overall, the tetracistronic trVLP assay represents the most comprehensive lifecycle modeling system for Ebola viruses available to date, and allows the modeling of genome replication and transcription, particle morphogenesis and budding, as well as attachment and entry into target cells over multiple infectious cycles. As such, it has tremendous potential for use in investigating the biology of Ebola viruses under BSL2 conditions.
Disclosures

The authors declare that they have no competing financial interests.

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