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Reverse Genetics Approaches to Control Arenavirus

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Abstract

Several arenavirus cause hemorrhagic fever disease in humans and pose a significant public health problem in their endemic regions. To date, no licensed vaccines are available to combat human arenavirus infections, and anti-arenaviral drug therapy is limited to an off-label use of ribavirin that is only partially effective. The development of arenavirus reverse genetics approaches provides investigators with a novel and powerful approach for the investigation of the arenavirus molecular and cell biology. The use of cell-based minigenome systems has allowed examining the *cis*- and *trans*-acting factors involved in arenavirus replication and transcription and the identification of novel anti-arenaviral drug targets without requiring the use of live forms of arenaviruses. Likewise, it is now feasible to rescue infectious arenaviruses entirely from cloned cDNAs containing predetermined mutations in their genomes to investigate virus-host interactions and mechanisms of pathogenesis, as well as to facilitate screens to identify anti-arenaviral drugs and development of novel live-attenuated arenavirus vaccines. Recently, reverse genetics have also allowed the generation of tri-segmented arenaviruses expressing foreign genes, facilitating virus detection and opening the possibility of implementing live-attenuated arenavirus-based vaccine vector approaches. Likewise, the development of single-cycle infectious, reporter-expressing, arenaviruses has provided a new experimental method to study some aspects of the biology of highly pathogenic arenaviruses without the requirement of high-security biocontainment required to study HF-causing arenaviruses. In this chapter we summarize the current knowledge on arenavirus reverse genetics and the implementation of plasmid-based reverse genetics techniques for the development of arenavirus vaccines and vaccine vectors.

Keywords

Recombinant arenavirus; Arenavirus rescue systems; Arenavirus reverse genetics; Arenavirus GP-expressing cells; Reporter genes; Minigenome assays; Tris-segmented arenavirus; Single-cycle infectious arenavirus; Virus vectors; Reporter virus; Vaccines; Antiviral drugs

1 Introduction

1.1 Arenaviruses and Their Impact on Human Health

Arenaviruses cause chronic infections in rodents with a worldwide distribution [1]. Asymptomatically infected animals move freely in their natural habitat and may invade human dwellings. Humans are infected most likely through mucosal exposure to aerosols, or by direct contact between infectious materials and abrade skin. These infections are common and in some cases severe [1].

The family *Arenaviridae* consists currently of two genera: (1) *Mammarenavirus* with 25 recognized virus species that are classified into two distinct groups, Old World (OW) and New World (NW) arenaviruses [1], and (2) *Reptarenavirus*, a new genus that has been established to accommodate the recently discovered snake arenaviruses [2–5]. Classification of members of the genus *Mammarenavirus* was originally established based on serological cross-reactivity, but it is well supported by recent sequence-based phylogenetic studies [1]. Genetically, OW arenaviruses constitute a single lineage, while NW arenaviruses segregate into clades A, B, A/B, and C [1]. The OW arenavirus, Lassa virus (LASV), causes hemorrhagic fever (HF) disease in humans, which represents a serious public health problem in its endemic areas of West Africa [1, 6–10]. LASV is estimated to infect several hundred thousand individuals yearly in its endemic regions of West Africa resulting in a high number of Lassa fever (LF) cases associated with high morbidity and significant mortality. Moreover, increased travel has led to the importation of cases of Lassa fever into the USA, Europe, Japan, and Canada [11–13]. Moreover, recent studies indicate that LASV endemic regions are expanding [14] and the association of Lujo virus (LUJV) [15], a newly identified OW arenavirus associated with an outbreak of HF in Southern Africa in 2008, has raised concerns about the emergence of novel HF OW arenaviruses outside their current known endemic regions. On the other hand the NW arenavirus Junin virus (JUNV), endemic to the pampas of Argentina, causes Argentine HF (AHF) with a high (15–30 %) case fatality rate [6]. Likewise, NW arenavirus Machupo virus (MACV) [16, 17] and Chapare virus (CHPV) [18], Sabia virus (SABV) [19, 20], Guanarito virus (GTOV) [21–23], Whitewater Arroyo virus (WWAV) [24, 25], and Ocozocoautla de Espinosa virus (OCEV) [26] are responsible for causing HF in Bolivia, Brazil, Venezuela, the USA, and Mexico, respectively. Moreover, mounting evidence indicates that the worldwide-distributed prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) is a neglected human pathogen of clinical significance, especially in cases of congenital infection [27–31]. In addition, LCMV poses a special threat to immunocompromised individuals, which has been illustrated by fatal cases of transplant-associated infections by LCMV [32–34]. OW arenaviruses LASV and LUJV and NW arenavirus JUNV, MACV, GTOV, and CHPV have features that make them credible biodefense threats and have been included by the National Institute of Allergy and Infectious Diseases (NIAID) as Category A biological agents that pose a significant biodefense concern [35]. Concerns about human pathogenic arenavirus infections are aggravated by the lack of Food and Drug Administration (FDA)-licensed vaccines and antiviral drug treatment being limited to the use of ribavirin (Rib) [36] that is only partially effective [37–39]. Evidence indicates that morbidity and mortality of LASV infections, and likely other HF arenavirus infection, are associated with, at least partly, the failure of the host's innate immune response to restrict virus replication and to facilitate the initiation of an effective adaptive immune response [9]. Accordingly, viremia is a highly predictive factor for the outcome of LF patients [9]. Therefore, therapeutic interventions resulting in reduced virus load, despite lack of virus clearance, are expected to promote the recovery of appropriate host defense responses to control arenavirus multiplication and associated disease.

1.2 Current Strategies to Combat Human Arenavirus Infections

1.2.1 Arenavirus Vaccines—The live-attenuated Candid#1 strain of JUNV induces a strong neutralizing antibody response in several animal model systems, and it has been

shown to be an effective vaccine to combat AHF in humans without causing serious adverse effects [6, 40, 41]. However, the Candid#1 vaccine is licensed only as an investigational new drug (IND) in the USA and studies addressing the stability of its attenuation, long-term immunity, and safety have not been conducted. Moreover, Candid#1 does not protect against LASV (Dr. Paessler and colleagues, personal communication), the HF arenavirus with the highest impact in public health [1, 6–10]. Despite significant efforts dedicated to the development of LASV vaccines, not a single LASV vaccine candidate has entered a clinical trial but the MOPV/LASV reassortant ML29 as well as recombinant vesicular stomatitis virus (VSV) and vaccinia virus expressing LASV antigens have shown promising results in animal models, including nonhuman primates (NHP), of LASV infection [42]. However, the high prevalence of HIV within LASV-endemic regions in West Africa raises safety concerns about the use of VSV- or vaccinia-based platforms. Likewise, the mechanisms of ML29 attenuation remain poorly understood and additional mutations in ML29 or reassortants between ML29 and circulating virulent LASV strains could result in viruses with enhanced virulence.

The recent development of reverse genetics systems for JUNV [43, 44] and LASV [45, 46] would facilitate the elucidation of the genetic determinants of JUNV and LASV virulence. This, in turn, should help with the design of safer live-attenuated arenavirus vaccines by minimizing concerns related to reversion of virulence, establishment of persistent infection, and in general conditions associated with high risk for replicating viruses including immunocompromised individuals.

1.2.2 Arenavirus Antiviral Drugs—In vitro and in vivo studies have documented the prophylactic and therapeutic value of the nucleoside analogue Rib (1-b-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) against several arenaviruses [36]. Moreover, Rib has been shown to reduce significantly both morbidity and mortality associated with LASV infection in humans [36, 47], and experimentally in MACV [48] and JUNV [49] infections, if given early in the course of clinical disease. The mechanisms by which Rib exerts its anti-arenaviral action remain poorly understood, but likely involve targeting different steps of the virus life cycle [50, 51]. Recent evidence indicates that Rib can be used as substrate by the RNA-dependent RNA polymerase (RdRp) of some riboviruses leading to C to U and G to A transitions [52, 53], and this mutagenic activity of Rib has been linked to its antiviral activity via lethal mutagenesis. However, Rib was also shown to strongly inhibit LCMV replication without exerting any noticeable mutagenic effect on the viral genome RNA [54]. Anemia and congenital disorders are two significant side effects associated with the use of Rib. In addition, oral Rib is significantly less effective than the one administered intravenously, which poses logistic complications in regions with limited clinical infrastructure [37–39].

Several inhibitors of inosine-5'-monophosphate (IMP) dehydrogenase, as well as acyclic and carbocyclic adenosine analogue inhibitors of the S-adenosylhomocysteine (SAH) hydrolase, have been shown to have also anti-arenavirus activity [36]. Likewise, the pyrimidine biosynthesis inhibitor A3, that exhibits broad-spectrum antiviral activity against negative- and positive-sense RNA viruses, retroviruses, and DNA viruses [55], has been shown to be more efficient than Rib in controlling arenavirus multiplication and this

inhibitory effect is due, at least in part, to its ability to interfere with viral RNA replication and transcription [56]. Moreover, since Rib and A3 target different metabolic pathways within the cell, they are excellent candidates for combination anti-arenaviral therapy to circumvent some limitations of Rib monotherapy [56]. A variety of sulfated polysaccharides, phenothiazines, and myristic acid have been reported to have activity against several arenaviruses [36]. However, in general these compounds displayed only modest and rather nonspecific effects often associated with significant toxicity. Nevertheless, favipiravir, a broad-spectrum RdRp inhibitor, has shown promising results including some protection (20 % survival) in a guinea pig model of fatal AHF [57, 58]. Recently a high-throughput screening (HTS) based on a virus-induced cytopathic effect (CPE) assay successfully identified a potent small-molecule inhibitor of HF NW arenaviruses that acts by targeting the viral GP [59]. Likewise, cell-based HTS based on the use of pseudotyped virion particles bearing the GP of highly pathogenic arenaviruses identified several small-molecule inhibitors of virus cell entry mediated by LASV GP [60]. These findings illustrate how complex chemical libraries used in the context of appropriate screening assays can be harnessed into a powerful tool to identify candidate antiviral drugs with highly specific activities. To achieve this goal, recent progress in the molecular and cell biology of arenaviruses including the development of reverse genetics systems has opened new avenues for the identification of steps in the arenavirus life cycle that are amenable to drug targeting, and the development of appropriate screening strategies to identify such drugs.

1.3 Arenavirus Virion Structure and Genome Organization

Arenavirus are bi-segmented negative-sense, single-stranded, RNA viruses that belong to the *Arenaviridae* family [1]. Each arenaviral segment encodes, using an ambisense coding strategy, two viral proteins in opposite orientation separated by a noncoding intergenic region (IGR) between the two viral genes (Fig. 1a) [1]. The large (L) segment encodes the viral RdRp or L polymerase protein (Fig. 1a, blue) involved in viral replication and gene transcription [61], and, in opposite direction, the small really interesting new gene (RING) finger protein Z (Fig. 1a, orange) that is the counterpart of the matrix (M) protein present in other negative-stranded (NS) RNA viruses and the major driving force of arenavirus budding and assembly [62–64]. The small (S) segment encodes for the viral glycoprotein precursor (GP) (Fig. 1a, green) that is posttranslationally cleaved to form the two mature virion glycoproteins (GP1 and GP2) involved in receptor binding, cell entry, and fusion [65–67], and the viral nucleoprotein (NP) (Fig. 1a, red), which encapsidates the viral RNA and, together with the polymerase L and the viral RNA, constitutes the viral ribonucleoproteins (vRNPs) that are the minimal factors involved in arenavirus genome replication and gene transcription [1, 68, 69]. In addition, arenavirus NP mediates the incorporation of the vRNPs into mature infectious virions by interacting with Z [70]. Furthermore, NP has also been shown to counteract the cellular host type-I interferon (IFN-I) [71–76] and inflammatory [75, 77] responses during viral infection.

1.4 Arenavirus Life Cycle

Arenavirus replication cycle takes place entirely in the cytoplasm of infected cells [1]. Homo-trimer complexes, consisting of the GP1 globular head and GP2 stalk region, form the spikes that decorate the surface of the arenavirus envelope [1, 78] (Fig. 1b). GP1, located at

the top of the spike, mediates attachment of the virus particle to receptors located in the surface of the cell [79]. Alpha-dystroglycan (α DG) has been described as the main receptor for OW and NW clade C arenaviruses [80–82]. However, clade A, B, and A/B NW arenaviruses appear to use the human transferrin receptor I as the cellular receptor for viral entry [83]. Upon viral attachment, arenavirus virions enter the cell via receptor-mediated endocytosis [79, 84]. Fusion of the virion and the endosome membranes is triggered by acidification of the endosome, which induces a conformational change in the viral GP1/GP2 complex [84]. Fusion of the viral and cellular membranes releases the vRNPs into the cytoplasm of infected cells, where viral RNA replication and gene transcription occur [1].

Arenavirus gene transcription is mediated by the viral promoters located within the untranslated regions (UTRs) at the 3' termini of viral (v)RNA and complementary (c)RNA species [1] (Fig. 2). NP and L proteins, located at the 3' end of the S and L viral segments, respectively, are translated from mRNAs with anti-genomic sense polarity transcribed directly from the vRNAs and, therefore, are the first arenaviral proteins encoded upon infection [1] (Fig. 2). Transcription termination is mediated by a secondary stem-loop structure formed by the IGR found in both vRNA segments, between each of the two viral genes [1], that function as a bona fide transcription termination signal for the virus polymerase complex (Fig. 2). GP and Z proteins, on the other hand, are located, respectively, at the 5' end of the S and L viral segments and are not translated directly from mRNA derived from the vRNAs but from antigenome complementary RNA species after replication of the vRNAs [1] (Fig. 2). Complementary RNA segments also serve as templates for the synthesis of nascent vRNAs [1]. Newly synthesized vRNAs are encapsidated by the viral NP to form the vRNP complexes and are packaged into progeny infectious virions by interaction of the viral Z [70]. Arenavirus assembly involves the interaction of the new vRNP complexes with the GP1/GP2 complexes present in the membrane of infected cells, a process mediated by interaction with the Z protein [85]. Newly synthesized and assembled virions bud from infected cells, a process mediated by the Z protein, and are released from infected cells to infect new cells [62, 63, 86, 87].

1.5 Reverse Genetics Approaches for the Investigation of the Molecular and Cellular Biology of Arenavirus

The introduction of reverse genetic approaches to rescue infectious recombinant arenaviruses from plasmid DNA had significantly advanced the arenavirus field and had led to many important discoveries surrounding arenavirus biology [1, 61]. Reverse genetics systems had facilitated the investigation of the *cis*-acting and the *trans*-acting factors that control the replication cycle of arenaviruses, including viral cell entry, genome viral replication, and gene transcription, assembly, and budding [88–91]. Reverse techniques have also allowed the generation of recombinant arenaviruses with mutations in their genomes to examine their contribution in viral replication using cell cultures as well as in viral pathogenesis and associated disease using validated animal models of infection [72, 92–95]. Likewise, implementation of arenavirus reverse genetics has allowed researchers to study arenavirus-host interactions [90, 96, 97], and potentiated the generation of novel candidate live-attenuated arenavirus vaccines [91, 96–100] as well as the development of screening methods to identify and evaluate novel anti-arenavirus drugs targeting specific steps of the

virus life cycle [96, 99]. Reverse genetics of arenaviruses have also been used for the generation of recombinant tri-segmented (r3) arenavirus expressing additional genes of interest (GOI) [91, 96, 97, 100, 101] to facilitate the study of several members in the family, including the identification of antivirals inhibiting the replication cycle of arenaviruses [56] as well as their possible implementation as vaccine vector candidates [91, 96, 97, 101]. Finally, arenavirus reverse genetics have been used for the generation of a single-cycle infectious, reporter-expressing, recombinant arenaviruses that are limited to replicate in GP-expressing complementing cell lines [99, 102], allowing the study of some aspects of the biology of highly pathogenic arenaviruses (e.g., neutralizing antiviral responses and identification of inhibitors of GP-mediated cell entry) without the use of special biosafety conditions [99]. In this chapter we focus on arenavirus reverse genetics techniques, based on human cells, for the prototype member in the family, LCMV [70, 91, 96, 97, 103]. Generation of recombinant LCM viruses can be performed under biosafety level (BSL)-2 conditions. Rescue of other arenaviruses may require higher BSL facilities and appropriate safety and security measures may be adopted [104].

2 Materials

2.1 Plasmids for the Generation of Recombinant Arenaviruses

The plasmids used for the generation of recombinant arenaviruses can be transformed and amplified in DH5 α competent cells and grown in LB media at 37 °C for 16–18 h, with the exception of the pPol-I L plasmid (*see* Note 1). Plasmids can be prepared using commercially available maxiprep kits following the manufacturer's recommendations. Plasmids can be stored at –20 °C until use (*see* Notes 2 and 3).

2.1.1 pCAGGS Protein Expression Plasmids—The pCAGGS protein expression plasmid uses the chicken β -actin promoter and the rabbit β -globin polyadenylation (pA) signal sequence [105] to direct the synthesis of arenavirus NP and L polymerase, which are the minimal components for viral gene transcription and genome replication [56, 61, 69, 70, 91, 92, 96–98] (Fig. 3a).

The pCAGGS plasmid expressing LCMV GP is required to initiate the first round of virus production for the rescue of single-cycle LCMV [99, 102].

A pCAGGS plasmid expressing the secreted luciferase from the ostracod *Cypridina noctiluca* (Cluc) is used in the minigenome (MG) experiments (Fig. 4) to normalize transfection efficiencies [56, 91, 96, 98] (*see* Notes 4 and 5).

¹We recommend growing the pPol-I L plasmid at 30 °C for 24 h.

²Plasmid concentrations can be determined using spectrophotometry at 260 nm, with DNA purity being estimated using the 260:280 nm ratio (it is optimal to reach a 1.8–2.0 ratio for optimal arenavirus rescue).

³All plasmids can be generated using standard cloning techniques and sequenced using standard protocols.

⁴We use a secreted luciferase, so both the MG *Gaussia* luciferase (Gluc) and Cluc can be measured directly from tissue culture supernatants (TCS) [56, 91, 96, 98, 145] (Fig. 4).

⁵Other polymerase II (Pol-II)-driven expression plasmids can be used instead of pCAGGS for arenavirus MG and rescue approaches. Likewise, other alternative reporter luciferase genes can be used for MG assays to normalize transfection efficiencies.

2.1.2 pPol-I vRNA Expression Plasmids—Arenavirus reverse genetic approaches were originally developed with the use of the mouse RNA polymerase I (Pol-I) promoter to direct intracellular synthesis of S and L genome, or antigenome, RNA species [106]. However, unlike the Pol-II promoter, the transcriptional activity of the Pol-I promoter exhibits stringent species specificity [96, 107] (*see Note 6*).

To evaluate viral genome replication and gene transcription, a vRNA-like MG plasmid is used (pPol-I MG) (Fig. 4) [56, 69, 70, 91, 92, 96–98]. In this plasmid, the viral GP and NP open reading frames (ORFs) are replaced by reporter genes. In our case, we replaced GP for Gluc and NP for the green fluorescent protein (GFP) (Fig. 4) (*see Notes 7 and 8*).

For the generation of recombinant wild-type (WT) bi-segmented arenaviruses (Fig. 5), two pPol-I plasmids expressing the S (pPol-I S) and L (pPol-I L) viral genomic or antigenomic RNAs are used (Fig. 3b) [56, 69, 70, 91, 92, 96–98].

To generate r3 arenavirus (Fig. 6), the pPol-I plasmid expressing the S vRNA segment is separated into two (S1 and S2) plasmids (Fig. 3c) [91, 96, 97, 100]. In the pPol-I S1 plasmid, the viral NP is replaced by GFP (or an alternative reporter or foreign GOI) and in the pPol-I S2 plasmid, the viral GP is replaced by Gluc (or an alternative reporter or foreign gene) (Fig. 3c) [91, 96, 97] (*see Note 9*).

For the generation of a single-cycle infectious arenavirus (Fig. 7), the viral GP is substituted with GFP (or an alternative reporter or foreign GOI) within the pPol-I S vRNA (pPol-I S GP/GFP) (Fig. 7a) [99, 102].

To generate recombinant arenaviruses use the two-plasmid-based approach (Fig. 8) [96], plasmids containing both the RNA Pol-II and -I promoters, to allow for Pol-II-mediated expression of the minimal viral *trans*-acting factors NP and L required for viral genome replication and gene transcription, and Pol-I to mediate initial intracellular synthesis of the S and L vRNA genomic or anti-genomic segments (Fig. 3d) [96] (*see Note 10*).

2.2 Cell Lines for the Generation of Recombinant Arenaviruses

As discussed above, the gold standard to assess virus replication and transcription using the MG approach or to rescue recombinant arenaviruses relies on the mouse Pol-I promoter and rodent BHK-21 cells, as these cell lines are easy to maintain, have high transfection efficiencies, and are able to produce high viral titers [91, 96, 97, 100]. However, BHK-21

⁶If arenavirus MG or rescue attempts are going to be performed in murine cells, the pPol-I vRNA expression plasmids should contain the murine Pol-I promoter [96, 106]. Alternatively, if human 293 T or monkey Vero cells are used to assess viral replication and gene transcription (MG assay) or generation of recombinant viruses, the pPol-I plasmids should be used to drive the expression of the arenavirus vRNAs under the human Pol-I promoter [56, 91, 96–98].

⁷Alternative reporter genes can be used to evaluate viral replication and gene transcription using these MG approaches.

⁸The viral GP can be replaced by GFP and NP by Gluc [91]. It is important to state that reporter gene expression will depend on their location in the vRNA-like MG plasmid since it has been shown higher expression levels of arenavirus NP than GP during viral infection and MG transfections [91].

⁹As indicated above, arenavirus NP expression levels are higher than those of the viral GP [91] during viral infection and therefore reporter or foreign GOI expression will depend on their location in the viral S segment [91].

¹⁰To avoid possible recombination that could occur by including the sequence of the same viral protein twice in the same plasmid, we introduced the NP ORF within the plasmid containing the L vRNA segment (pCAGGS NP/pPol-I L) and the L polymerase ORF within the plasmid containing the S segment (pCAGGS L/pPol-I S) (Figs. 3d and 6) [96].

cells do not meet guidelines set forth by the FDA for human vaccine generation due to the presence of tumorigenic agents that may affect the purity of the vaccine seed [96]. Vero cells, on the other hand, have received approval from the FDA for the generation of vaccine seeds [96, 97, 108]. While Vero cells are easy to maintain and produce high viral titers, the transfection efficiencies in Vero cells are generally lower and thus may lead to difficulties in rescuing recombinant arenaviruses [96]. Alternatively, human 293 T cells can be used to evaluate MG activities or to generate recombinant arenaviruses [96] (*see Note 11*). All cells are grown at 37 °C in a 5 % CO₂ atmosphere.

Vero cells: African green monkey kidney epithelial cells available from the American Type Culture Collection (ATCC).

Human 293 T cells: Human embryonic kidney cells available from the ATCC.

BHK-21 cells: Baby hamster kidney cells available from the ATCC.

GP-expressing BHK-21 or Vero cells: To generate GP-deficient, reporter-expressing, recombinant LCMV (rLCMV GP/GFP), BHK-21 or Vero cell lines constitutively expressing LCMV GP are generated by co-transfection of the LCMV GP-expressing pCAGGS protein expression plasmid, together with a hygromycin (or other drug) resistance pCB7 plasmid, using Lipofectamine 2000 (LPF2000) [99, 102]. After transfection, cells are plated into 10 cm dishes at low density and incubated in the presence of normal media supplemented with hygromycin B (250–500 µg/ml) [92, 99, 109, 110]. Individual cell clones are isolated using cloning rings or any other selection method (e.g., fluorescence-activated cell sorting, FACS) and evaluated for LCMV GP expression using immunofluorescence, flow cytometry, or western blot approaches with appropriate antibodies [92, 99, 109, 110] (*see Note 12*). Uniformly (~85–95 %) LCMV GP-expressing cell clones are then evaluated for complementation of the rLCMV GP/GFP, using parental cells as negative controls [99, 102]. Transiently transfected GP-expressing cells should be used as internal positive controls. Constitutively LCMV GP-expressing, rLCMV GP/GFP-complementing, cell clones should be maintained in normal media supplemented with hygromycin B [99, 102] (*see Note 13*).

2.3 Tissue Culture Media and Reagents

DMEM 10 % FBS, 1 % PS: This media is used for maintenance of BHK-21, 293 T, and Vero cells [97]. To prepare this media, mix 445 ml Dulbecco's modified Eagle's medium (DMEM), 50 ml of fetal bovine serum (FBS), and 5 ml of 100× penicillin/streptomycin (PS) [56, 69, 70, 91, 92, 96–98]. DMEM 10 % FBS 1 % PS supplemented with 250–500 mg/ml

¹¹It is important to choose the proper cell line to successfully evaluate viral MG activities and to generate recombinant arenaviruses using plasmid-based reverse genetics techniques [96]. One of the advantages of using human 293 T in MG approaches is the identification of anti-arenavirals for their potential use in humans [96].

¹²We recommended the use of immunofluorescence or flow cytometry approaches to evaluate the percentage of LCMV GP-expressing cells.

¹³Alternatively to the generation of stable LCMV GP-expressing cells, rLCMV GP/GFP complementation can be achieved by transiently transfecting the pCAGGS LCMV GP expression plasmid 24 h prior to infection [110].

of hygromycin B is used for maintenance of GP-expressing stable cell lines [92, 99, 109, 110]. Store at 4 °C.

OptiMEM: OptiMEM media is used in the transfection protocol for MG assays and for the rescue of recombinant arenaviruses [56, 69, 70, 91, 92, 96–98].

Infectious media: This media is used during and after viral infections [97]. To prepare this media, mix (2:1) OptiMEM and DMEM 10 % FBS 1 % PS. Store at 4 °C [56, 69, 70, 91, 92, 96–98].

10× Phosphate-buffered saline (PBS): 80 g of NaCl, 2 g of KCl, 11.5 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 2 g of KH_2PO_4 . Add ddH₂O up to 1 L. Adjust pH to 7.3. Sterilize by autoclaving. Store at room temperature (RT). Working concentration should be prepared by diluting 10× PBS 1:10 with ddH₂O (1× PBS). Autoclave to sterilize and store at RT [56, 69, 70, 91, 92, 96–98].

2.5 % Bovine serum albumin (BSA): This is used as a blocking solution for immunofluorescence assays: 2.5 g of BSA in 97.5 ml of 1× PBS. Store at 4 °C [56, 69, 70, 91, 92, 96–98].

Lipofectamine 2000 (LPF2000): LPF2000 is used for plasmid DNA transfection in BHK-21, 293 T, or Vero cells. We recommend an LPF2000:DNA ratio of 2.5:1 for both MG and arenavirus rescue approaches (see Tables 1, 2, 3, and 4) [56, 69, 70, 91, 92, 96–98].

Trypsin-EDTA: Trypsin-EDTA is used to detach cell monolayer from the tissue culture plate, prior to DNA transfection or for the passaging of transfected cells [56, 69, 70, 91, 92, 96–98].

Biolux Gaussia Luciferase Assay kit: To measure Gluc activities from TCS in the MG assays or from r3 arenavirus-infected cells, we use the Biolux Gaussia Luciferase Assay kit [56, 69, 70, 91, 92, 96–98].

Biolux Cypridinia Luciferase Assay kit: To assess Cluc activities from TCS in the MG assay or from cells infected with r3 arenaviruses expressing Cluc, we used the Biolux *Cypridinia* Luciferase Assay kit [56, 69, 70, 91, 92, 96–98].

3 Methods

3.1 Arenavirus Minigenome (MG) Assays to Evaluate Viral Genome Replication and Gene Transcription

Arenavirus MG approaches have been developed to investigate the *trans*-acting factors and *cis*-acting sequences that control arenavirus genome replication and gene transcription [56, 61, 69, 70, 91, 92, 96–98]. Arenavirus MG systems have also allowed the identification of arenavirus L and NP as the minimal *trans*-acting factors required for arenavirus genome replication and gene transcription [61]. Likewise, MG approaches can be used to evaluate amino acid residues in NP and L critical for viral genome replication and gene transcription

[69, 70, 92, 96–98]. Moreover, MG approaches have been used for the identification of the genomic promoter recognized by the virus polymerase to initiate viral replication and gene transcription [111]. Implementation of MG approaches has also allowed the identification of arenavirus IGR as a bona fide transcription termination signal [112]. Furthermore, results from MG assays demonstrated that arenavirus Z has a dose-dependent inhibitory effect on both viral replication and gene transcription [113]. In addition, MG approaches were used to confirm the importance of both GP and Z for arenaviral genome packaging [68].

Other than understanding arenavirus biology, MG assay approaches have been instrumental in demonstrating the specificity and efficacy of siRNAs targeting viral mRNAs encoding NP and/or L [114]. In accordance, MG assays have also been used for the identification of anti-arenavirals targeting viral replication and transcription, as evidenced with the ribonucleoside analog Rib [58] and the pyrimidine biosynthesis inhibitor A3 [56]. A major advantage in using MG assays to identify effective therapeutics is that such approaches can be performed without the need of BSL-4 containment, an issue that bottlenecks most researchers from screening effective anti-arenavirals and specific siRNAs to target the human pathogenic arenaviruses of highest public health concern [104]. MG reverse genetics approaches have been developed, to date, for the OW LCMV [61, 91, 96–98], LASV [45, 46], and Lujo, LUJV [115] arenaviruses; and for the NW Tacaribe, TCRV [116], Junin, JUNV [43, 44, 96, 98], Pichinde, PICV [117], and Machupo, MACV [118] arenaviruses.

For arenavirus MG assays, susceptible cells are co-transfected with a Pol-I-driven plasmid regulating expression of a reporter-expressing vRNA-like segment (e.g., Gluc and GFP) together with the Pol-II-driven pCAGGS protein expression plasmids encoding the minimal *trans*-acting elements for replication and transcription (NP and L) [61], and a Pol-II-dependent Cluc to normalize transfection efficiencies (Fig. 4) [91, 96–98] (*see* Note 14). The pPol-I plasmid encodes the reporter genes in the place of the viral ORFs; thereby replication and transcription can be easily measured by reporter gene activity [91, 96–98]. To easily evaluate MG activities under a fluorescence microscope, we used a fluorescent protein (e.g., GFP). To quantify MG activities using luminometer, we used secreted forms of luciferase proteins (e.g., Gluc) [91, 96–98]. Since NP expression levels during viral infection are higher than those of GP, it is important to determine what reporter gene should substitute the viral ORFs [91]. In our case NP is substituted by GFP and GP is substituted by Gluc (Fig. 4) [97]. The other RNA sequences required for viral genome replication and gene transcription (e.g., UTRs and IGRs) are identical to those found in the viral S segment (Fig. 4). Efficient recognition of the viral-like reporter RNA by the viral NP and L requires precise vRNA 3' and 5' ends [44, 46, 106, 115, 119, 120]. Efficient vRNA 5' ends are obtained with the use of the Pol-I promoter [44, 106]. To obtain authentic vRNA 3' ends, both the self-cleaving hepatitis delta virus ribozyme (HDVR) and the Pol-I terminator (T) sequences can be used [96, 119, 120] (*see* Note 15).

¹⁴As indicated above, because of the species specificity of Pol-I promoters [96, 107], MG assays in murine and human cells should use murine and human Pol-I promoters, respectively [96, 107].

¹⁵We have found that the Pol-I T sequence is more efficient than HDVR sequences in producing arenavirus-like RNAs with the precise 3' ends in both MG and virus rescue approaches [96].

3.1.1 Arenavirus MG Experimental Approach (Fig. 4)

1. *OptiMEM-Lipofectamine 2000 (LPF2000)*: The MG assay can be performed in either 12- or 6-well plate, although the amount of DNA and the number of cells transfected should be adjusted accordingly (Table 1). Prepare 250 μ l of OptiMEM media with 3.75 or 7.5 μ g of LPF2000 (2.5 μ g LPF2000/ μ g plasmid DNA) per transfection in 12- or 6-well plates, respectively (Table 1) (*see* Notes 16 and 17). Incubate the OptiMEM-LPF2000 mixture for 5–10 min at RT. During this incubation time, prepare the OptiMEM-DNA plasmid mixture.
2. *OptiMEM-DNA plasmid*: In a separate tube, prepare the DNA plasmid mixture using the recommended amounts provided in Table 1 in a total volume of 50 μ l of OptiMEM media.
3. *Combine OptiMEM-LPF2000 and OptiMEM-DNA plasmid*: Pipette 250 μ l of OptiMEM-LPF2000 (**step 1**) into the OptiMEM-DNA (**step 2**) and incubate this mixture for 20–30 min at RT. During this incubation period, prepare the cells for transient transfection in suspension.
4. *Preparation of cells*: MG assays can be conducted in either human 293 T or rodent BHK-21 cells, depending on whether the MG reporter plasmid is regulated by the human or murine pPol-I promoters, respectively [96] (*see* Note 18). Prior to manipulating the cells, bring the 1 $\times$ PBS, DMEM 10 % FBS, 1 % PS media, and trypsin-EDTA mixture to 37 °C for ~10 min.
 - a. Wash the cells, twice, with 5 ml 1 $\times$ PBS.
 - b. Trypsinize cells using 1 ml trypsin-EDTA. Cell detachment is usually accomplished by incubating the cells in a humidified 37 °C, 5 % CO₂, chamber, for ~5 min. Gently tapping the tissue culture plate will expedite this process.
 - c. Carefully resuspend the cells in 9 ml of DMEM 10 % FBS, 1 % PS, once the cells have been fully detached. Place the cells in a 15 ml tube and centrifuge for 5 min at 1000 $\times$ g.
 - d. Remove the media and resuspend the cell pellet in 10 ml of fresh DMEM 10 % FBS, 1 % PS, and count the cells using a hemocytometer. Adjust the cell concentration to 10⁶ cells/ml. Successful MG assay is typically achieved by transfecting ~5–6 $\times$ 10⁵ (12-well plates) or ~1–2 $\times$ 10⁶ (6-well plates) cells per transfection (Table 1).

¹⁶We typically perform the MG assay in triplicate to get a good representation of the experiment and for statistical analysis.

¹⁷Transfection in the absence of the protein expression plasmid encoding for NP (pCAGGS NP) should be included as a negative control.

¹⁸We recommend transfecting cells that are more susceptible to LPF2000/DNA transfection, such as 293 T or BHK-21 cells, to improve the quality and reproducibility of the MG experiments.

5. *Mix LPF2000/DNA with cells:* After 20–30-min LPF2000/DNA incubation, pipette into each LPF2000/DNA tube (**step 3**) the appropriate concentration of cells (**step 4**). Let the LPF2000/DNA/cell mixture incubate for ~5 min at RT.
6. *LPF2000/DNA/cell plating:* Transfer the LPF2000/DNA/cell mixture into individual 12- or 6-well tissue culture plate. Gently tap the plate to distribute the cells uniformly and incubate the cells in a 5 % CO₂ humidified 37 °C incubator for ~6–12 h, after which the TCS is removed and replaced with 1 ml of fresh DMEM 10 % FBS, 1 % PS.
7. *Measure MG reporter gene activity:* After incubating the cells for a total of ~48 h, measure the MG reporter gene activity. Depending on the genes encoded by the MG plasmid, the assay can be determined qualitatively by fluorescence microscopy (e.g., GFP expression) or quantitatively using a luminometer (e.g., Gluc) (see Note 19).

3.2 Generation of Recombinant Arenaviruses

The ability to generate recombinant arenaviruses with specific mutations and study their phenotype in vitro and in vivo in their natural hosts has allowed researches to evaluate the contribution of viral determinants to the virus life cycle [89], virus-host interactions [90], mechanisms of pathogenesis [121], role of NP in the inhibition of the IFN-I response [72, 94], and potential development of live-attenuated arenavirus vaccines [69, 70, 92, 96–98]. Moreover, these reverse genetics techniques have allowed the generation of r3 arenaviruses expressing foreign GOI, opening the possibility of implementing live-attenuated arenavirus as vaccine vectors [91, 96, 97, 101]. Moreover, the recent development of single-cycle infectious, reporter-expressing, arenaviruses has provided a new experimental method to study some aspects of the biology of highly pathogenic members in the arenavirus family without the requirement of special biosafety conditions [104], currently required to study HF-causing members in the family [99].

Originally established for the prototyped member in the family, LCMV [106, 122], plasmid-based arenavirus reverse genetics techniques have expanded, to date, for the generation of OW LASV [45, 46] and LUJV [115] arenaviruses, and for the NW JUNV [43, 44], PICV [117], and MACV [118] arenaviruses. Initially, arenavirus reverse genetics approaches were developed based on the use of the bacteriophage T7 promoter [122]. However, current approaches for the generation of recombinant arenaviruses rely on the use of RNA Pol-I promoters to launch the intracellular synthesis of the S and L RNA genome or antigenome species, which are subsequently replicated and transcribed by the virus L and NP, the minimal viral *trans*-acting factors required for viral genome replication and gene transcription, encoded by RNA Pol-II-dependent promoter protein plasmids [106]. Although these techniques were established with the use of murine Pol-I promoters [106], the transcriptional activity of the RNA Pol-I exhibits species specificity preventing the

¹⁹The use of GFP and Gluc as reporter genes allows the measurement of MG activities at different times post-transfection using the same transfected cells [69, 70, 92, 96–98].

development of vaccine candidates that can be administered to humans [96, 107]. This barrier was recently solved by the implementation of human Pol-I promoters to drive vRNA expression, which allowed for the generation of recombinant OW (LCMV) and NW (Candid#1) arenaviruses from human 293 T and FDA-approved Vero cell lines [96, 97].

Originally established with the co-transfection of the two pCAGGS protein expression plasmid driven by the expression of the viral RdRp L and NP, together with the Pol-I-driven plasmids expressing the viral S and L segments, the ability to successfully generate recombinant arenaviruses has been reduced to two plasmids by combining within the same plasmid Pol-I-driven vRNA with Pol-II-driven protein constructs (Fig. 6) [96] (*see Note 20*).

3.2.1 Arenavirus Four-Plasmid Rescue System Experimental Approach (Fig. 5)

1. *OptiMEM-LPF2000*: Prepare 250 µl of OptiMEM media with 10 µg of LPF2000 (2.5 µg LPF2000/µg plasmid DNA) per transfection (Table 2a). Virus rescues are performed in 6-well plates (*see Note 21*). Incubate the OptiMEM-LPF2000 mixture for 5–10 min at RT. During this incubation time, prepare the OptiMEM-DNA plasmid mixture.
2. *OptiMEM-DNA plasmid*: In a separate tube, prepare the DNA plasmid mix using the recommended amounts provided in Table 2a in a total volume of 50 µl of OptiMEM media.
3. *Combined OptiMEM-LPF2000 and OptiMEM-DNA plasmid*: Pipette 250 µl of OptiMEM-LPF2000 (**step 1**) into the OptiMEM-DNA (**step 2**) and incubate this mixture for 20–30 min at RT. During this incubation period, prepare the cells for transient transfection in suspension.
4. *Preparation of cells*: Arenavirus rescues can be performed in either rodent (BHK-21), human (293T), or African green monkey kidney (Vero) cells, depending on the source of the Pol-I promoter in the vRNA expression plasmids. Before manipulating the cells, bring the 1× PBS, DMEM 10 % FBS, 1 % PS media, and trypsin-EDTA mixture to 37 °C for ~10 min.
 - a. Wash the cells, twice, with 5 ml 1× PBS.
 - b. Trypsinize cells using 1 ml trypsin-EDTA. Cell detachment is usually accomplished by incubating the cells in a humidified 37 °C, 5 % CO₂, chamber, for ~5 min. Gently tapping the tissue culture plate will uniformly detach the cells.
 - c. After cells are completely detached from the plate, carefully resuspend the cells with 9 ml of DMEM 10 % FBS, 1 % PS. Place the total media/cell mixture in a 15 ml

²⁰The benefit of performing arenavirus rescues using a two-plasmid approach is to increase successful co-transfection of cells that are poorly transfected, such as Vero cells, with the goal of vaccine implementation [96, 97].

²¹To increase the likelihood of successful virus rescue, virus rescues are attempted in triplicate. Therefore, prepare enough OptiMEM-LPF2000 based on the number of virus rescues planned.

- centrifuge tube and centrifuge the cells for 5 min at $1000 \times g$.
- d. Remove the media and resuspend the cells in 10 ml of fresh DMEM 10 % FBS, 1 % PS, and count the cells using a hemocytometer. Adjust the cell concentration to $\sim 1.0\text{--}1.2 \times 10^6$ cells/ml (Table 2a).
5. *Mix LPF2000/DNA with cells*: After 20–30-min incubation, pipette into each LPF2000/DNA mixture tube (**step 3**) $\sim 1.0\text{--}1.2 \times 10^6$ cells/well (**step 4**). Let the LPF2000/DNA and cell mixture incubate for ~ 5 min at RT.
 6. *LPF2000/DNA/cell plating*: Transfer the LPF2000/DNA/cell mixture into individual wells of a 6-well tissue culture plate. Gently tap the plate to distribute the cells uniformly and incubate the cells in a 5 % CO_2 humidified 37°C incubator for $\sim 6\text{--}12$ h.
 7. *Transfection media change*: After $\sim 6\text{--}12$ -h incubation, replace the TCS from the transfection with 2 ml of infectious media and return the cells to the incubator and incubate for 48 h.
 8. *Cell passage*: After 2-day incubation, transfected cells should reach ~ 100 % confluence.
 - a. Remove TCS and gently wash the cells, twice, with $1 \times$ PBS.
 - b. Trypsinize cells by adding 500 μl of trypsin-EDTA/well. Return the cells to the incubator and let them incubate for ~ 5 min. Gently tap the cells to completely detach them from the plate.
 - c. Carefully resuspend the cells with 1 ml of DMEM 10 % FBS, 1 % PS, and transfer to a 1.5 ml microcentrifuge tube.
 - d. Centrifuge the cells for 5 min at $5000 \times g$, 4°C , in a microcentrifuge.
 - e. Remove the TCS and resuspend the cells in 1 ml of infectious media and transfer to a 10 cm tissue culture dish. Bring up the volume in the plate to 10 ml with infectious media. Gently shake the 10 cm tissue culture dish to allow uniform distribution of the cells and incubate the cells at 37°C , 5 % CO_2 , for 72 h.
 9. *Virus recovery from TCS*: After 3 days of incubation, collect the TCS from the 10 cm tissue culture plates (**step 8**) into a 15 ml centrifuge tube and centrifuge at $2500 \times g$, 4°C , for 5 min. Collect the TCS containing the virus and discard the cell pellet.

10. *Storage of virus:* Aliquot the virus in cryotubes and store them at -80°C (see Note 22). Virus can be stored at -80°C until confirmation of the presence of virus.

3.2.2 Two-Plasmid Arenavirus Rescue System Experimental Approach (Fig. 6)

—To generate recombinant arenavirus using the two-plasmid approach (Figs. 4d and 6), follow steps described above but using the proper DNA plasmid concentrations indicated in Table 2b [96, 97].

3.3 Generation of Recombinant Tri-Segmented (r3) Arenaviruses

Several approaches have been used to successfully generate recombinant NS RNA viruses expressing foreign genes. These include the use of the self-cleavage 2A protease of picornaviruses [123], the use of dicistronic genome segments containing internal promoters [124, 125], the use of internal ribosome entry sites (IRES) [126, 127], and the use of virus-specific packaging signals within vRNA segments [128, 129]. Although a viable strategy in other NS-segmented RNA viruses, these approaches were unsuccessful in yielding recombinant arenaviruses encoding foreign genes [100]. Successful rescue of r3 arenavirus packaging two S segments into mature, infectious virions has been described for the OW arenavirus LCMV [91, 96, 97, 100, 101] and the NW arenavirus JUNV [96, 97, 100]. Within this approach, the S segment is altered to replace one of the viral-encoded proteins (e.g., GP and NP) by a foreign GOI [91, 96, 97, 100, 101]. The physical separation of the GP and NP proteins into two different S segments (S1 and S2) represents a strong selective pressure to maintain a virus capable of packaging 1 L segment and 2 S segments. The ability of arenavirus to package 2 S segments has been previously suggested based on genetics [130] and structural [131, 132] analysis. Moreover, because of the stability of the r3LCM viruses, these findings suggest that production of infectious arenavirus particles containing two S and one L segments is a common event [1, 100]. Importantly, each of the S segments can direct expression of a GOI and therefore two foreign proteins can be expressed within the same virus, contrary to the situation observed with other NS-segmented RNA viruses. Notably, regulation of foreign protein expression depended on the location in the S segment [91]. Expression of a GOI in the NP locus is higher than that observed when the GOI was located in the GP locus, similar to the situation observed during viral infection [91]. Moreover, results obtained with the generation of r3 arenaviruses suggest that, unlike the situation observed with other NS-segmented RNA viruses [133] but similar to members in the bunyamwera family [134], the arenavirus NP- and GP-coding regions do not appear to play a critical role in the packaging of the viral S segment [100]. Several r3 arenaviruses have been generated that express one or two additional GOI [91, 96, 97, 100, 101]. Depending on the GOI expressed (protein function, size, etc.), these r3 arenaviruses showed little or no attenuation in cultured cells and they exhibited long-term genetic stability as reflected by unaltered expression levels during serial virus passages of the GOI incorporated into the S segments [100]. Although the r3 arenavirus rescue approach is as efficient as the WT bi-segmented and arenaviruses containing 2 S segments exhibit WT growth properties in vitro, including genetic and phenotypic stability, r3 arenaviruses exhibit, as compared to WT

²²Make small-volume aliquots to prevent multiple thaw cycles, which may reduce virus titers.

arenaviruses, attenuation in vivo [100]. Since r3 arenaviruses are not attenuated in vitro (ideal for vaccine production) but are attenuated in vivo, these r3 arenaviruses represent a great approach for their implementation not only as arenavirus vaccines but also as vaccine vectors [91, 96, 97, 101]. Importantly, the use of r3 arenaviruses expressing appropriate GOI (e.g., reporter genes) could be used to facilitate the identification of antiviral compounds or drugs amenable to HTS approaches or siRNA-based screening of libraries to identify host cell genes involved in the arenavirus life cycle [135].

To generate r3 arenaviruses, susceptible cells (e.g., murine or human cells) are co-transfected with the pCAGGS protein expression plasmids encoding NP and L together with the pPol-I L segment, and two pPol-I S segments, where the GP ORF is replaced with a foreign gene (pPol-I S1 NP/GOI1) and the NP ORF is replaced by another foreign gene in the second S segment (pPol-I S2 GOI2/GP) (Fig. 3c). So far, several r3 arenaviruses expressing CAT, fluorescent, and luciferase genes have been described [91, 96, 97, 100, 101].

3.3.1 R3 Arenavirus Rescue System Experimental Approach (Fig. 7)—R3 arenavirus rescue protocol is similar to the steps outlined above for WT bi-segmented arenaviruses. However, follow the proper plasmid DNA preparations and concentrations recommended in Table 3 [91, 96, 97, 100, 101].

3.4 Generation of Single-Cycle Infectious Recombinant Arenaviruses

Although replication-competent arenaviruses are the most biologically relevant systems for studying arenavirus biology, research with highly pathogenic HF-causing arenaviruses requires the use of BSL-4 containments [1]. Two OW arenaviruses (LASV and LUJV) and seven NW arenaviruses (JUNV [6], MACV [16, 17], CHPV [18], SABV [19, 20], GTOV [21–23], WWAV [24, 25], and OCEV [26]) are known to cause HF disease in humans. Accordingly, they are classified as NIAID Category A priority pathogens. HF-causing arenavirus research has been hampered by the requirement of BSL-4 facilities to handle live forms of these viruses [104]. Research with HF-causing arenaviruses could be facilitated by the development and implementation of valid surrogate systems that could be used under less strict biosafety conditions to circumvent the intrinsic complications associated with the use of BSL-4 facilities. These include, among others, disposal of biological waste, costs associated with BSL-4 facilities, safety, and expression of reporter genes as a valid surrogate to detect and quantify arenavirus infections [104]. Arenavirus GP-pseudotyped retroviruses or lentivirus approaches have been used instead to evaluate arenavirus cell entry [136, 137]. However, these approaches are limited to the investigation of arenavirus cell entry without addressing the contribution of other viral proteins (L, NP, and Z) to viral fitness, including the identification of antivirals targeting multiple steps in the replication cycle of arenaviruses. Generation of single-cycle infectious arenavirus offers the advantage of using a bona fide arenavirus and therefore would recreate all the steps of the arenavirus life cycle in GP-complementing cells, making this system more suitable for the screening of inhibitors targeting other steps than arenavirus cell entry [99]. Moreover, expression of the reporter gene facilitates its implementation to the HTS of libraries of compounds where expression of the reporter gene can be used as a valid surrogate for efficient viral replication [99].

Several single-cycle infectious viruses have been documented, including BSL-2 (rabies virus, influenza viruses, herpes simplex virus, and simian immunodeficiency virus) [109, 138–140], BSL-3 (influenza viruses, West Nile virus, and Kunjin virus) [110, 141, 142], and BSL-4 (Ebola virus) [143] viral pathogens. These single-cycle infectious viruses have been shown to be safe candidates for the identification of neutralizing antibodies and antivirals, and for studying other aspects of viral replication cycle. Because of the advantages associated with this approach, a replicating-competent, single-cycle infectious, recombinant LCMV where GFP substitutes for the viral GP gene (rLCMV GP/GFP) was generated [99, 102]. Replication of the rLCMV GP/GFP was restricted to cells expressing LCMV GP. Moreover, by generating constitutively expressing OW or NW arenavirus GP-expressing stable cell lines, rLCMV GP/GFP can be used to generate rLCMV GP/GFP pseudotyped with GPs from other members in the *Arenaviridae* family for the safe identification of neutralizing antibodies and inhibitors of arenavirus GP-mediated cell entry without the need for BSL-4 facilities [99].

Generation of single-cycle arenaviruses requires the co-transfection of susceptible cells with pPol-I L, pPol-I S GP/GFP (where the GP ORF is replaced by GFP) (Fig. 8a), and pCAGGS protein expression plasmids NP and L for replication and transcription, together with the pCAGGS protein expression plasmid encoding GP to initiate the first round of virion production (Fig. 8b) [99]. While infectious virions can be recovered from these transfected cells, these viruses will be able to infect cells but not produce infectious particles, unless they are complemented *in trans* by constitutively GP-expressing cells [99, 102]. Using similar stable or transient transfection strategies, the single-cycle infectious rLCMV GP/GFP can also be used to infect cells expressing the surface GP from other OW or NW arenaviruses to generate GP-pseudotyped rLCMV GP/GFP viruses (Fig. 7c) [99] (see Note 23).

3.4.1 Single-Cycle Infectious Recombinant Arenavirus Experimental Approach

(Fig. 8)—Generation of single-cycle infectious rLCMV GP/GFP is similar to the steps outlined above for LCMV WT, following the DNA plasmid and LPF2000 concentrations included in Table 4 [99]. Once generated, the growth properties of rLCMV GP/GFP should be evaluated in both parental and GP-expressing cells to confirm the single-cycle nature of the virus [99, 102]. To generate GP-pseudotyped rLCMV GP/GFP with arenavirus GPs of interest, constitutively expressing or transiently transfected GP cells are infected with the rLCMV GP/GFP at low multiplicity of infection, MOI (0.01), and incubated for 48–72 h [99]. Viral titers in TCS can be assessed by infecting GP-expressing cell lines and observe for fluorescent (GFP) loci at ~18–24 h post-infection [99].

3.5 Confirmation of Successful Recombinant Arenavirus Rescue

3.5.1 Wild-Type (WT) Arenavirus—Arenaviruses do not display classic cytopathic effect (CPE) observed with other NS RNA viruses [1]. Thus, successful rescue of WT arenaviruses

²³Transient transfection with GP is likely to vary in efficiencies and GP expression levels and, therefore, rLCMV GP/GFP stocks. Thus, we recommend the use of GP-stable expressing cells for both the generation of rLCMV GP/GFP and GP-pseudotyped rLCMV GP/GFP [99, 102].

must be evaluated by performing classical plaque assays [144] or by immunofluorescence using arenavirus-specific antibodies [56, 69, 70, 91, 92, 96–98] (*see* Notes 24 and 25).

Experimental Approach

1. A day before confirming viral rescue or titration, trypsinize Vero cells from 10 cm dishes as above. In this case, adjust the cell density to 2×10^5 cells/ml. Seed the cells in 96-well plates (100 μ l/well) and gently tap the plate so that a uniform cell monolayer of 80–90 % confluence ($\sim 4 \times 10^4$ cells/well) is reached the next day upon culturing the cells at 37 °C, 5 % CO₂.
2. On the day of virus titration, serially dilute (tenfold dilutions) viral TCS recovered from transfected cells in OptiMEM.
3. Remove the media and wash Vero cells twice with 50 μ l of 1 $\times$ PBS. Infect the cells with 50 μ l of the serially diluted virus. Allow virus adsorption at 37 °C, 5 % CO₂, for 90 min, rocking the plates every 15 min to allow uniform virus infection of the cell monolayer.
4. After 90-min infection, remove the viral inoculum and add 100 μ l of infectious media. Allow the cells to incubate for 16–18 h (*see* Note 26).
5. Remove the TCS, and fix and permeabilize the cells with 4 % formaldehyde and 0.1 % triton X-100 diluted in 1 $\times$ PBS for 15 min at RT (*see* Note 27).
6. Remove the fixation/permeabilization solution and wash the cells, three times, with 1 $\times$ PBS.
7. Block the cells with 2.5 % BSA in 1 $\times$ PBS for 1 h at RT (*see* Note 28).
8. During cell blocking, prepare the primary antibody. Antibodies specific to viral antigen should be diluted in blocking solution (2.5 % BSA), and centrifuge for 15 min at 3500 $\times g$ before use (*see* Note 29).
9. After blocking the cells, remove the blocking solution and incubate the cells with 50 μ l of the primary antibody at 37 °C, 5 % CO₂, for 1 h.
10. After 1-h incubation with the primary antibody, wash the cells three times with 1 $\times$ PBS, and incubate with 50 μ l of the fluorescein-conjugated

²⁴Because of the long incubation period (5–6 days) and the need of immunostaining arenavirus plaques in Vero cells, we recommend the use of immunofluorescence assays (~16–18 h) for confirmation of recombinant arenavirus rescues and for viral titrations [56, 69, 70, 91, 92, 96–98].

²⁵We usually evaluate viral titers in triplicates, using 96-well plates.

²⁶Viral infection lasting over 18 h post-infection may lead to secondary infections and, therefore, result in over-estimation of viral titers.

²⁷Alternatively you can fix the cells with 4 % formaldehyde diluted in 1 $\times$ PBS for 15 min at RT, before permeabilizing the cells with 0.1 % triton X-100 for 15 min at RT.

²⁸Alternatively cells can be blocked ON at 4 °C.

²⁹An antibody specific to arenavirus NP is recommended, as NP is the most abundantly produced viral protein in infected cells and will assist in easy detection of the virus [1]. The species (e.g., mouse, rabbit, etc.), nature (e.g., monoclonal or polyclonal), and proper dilution of the antibody used for viral detection and/or titration should be determined previously. For LCMV, we use the mouse monoclonal antibody 1.1.3 from hybridoma TCS diluted in 2.5 % BSA.

secondary antibody diluted (following manufacturer recommendations) in blocking solution (2.5 % BSA) at 37 °C, 5 % CO₂, for 30 min (see Note 30).

11. Following the 30-min incubation, remove the secondary antibody and wash the cells three times with 1× PBS.
12. At this moment, arenavirus rescue or titers can be determined under a fluorescent microscope. Viral titration is calculated by counting the fluorescent focus-forming units (FFU) and the respective dilutions.

3.5.2 Tri-Segmented (r3) Arenavirus—R3 arenaviruses typically encode for two foreign genes, such as fluorescent or luminescent reporter genes [91, 96, 97, 100, 101]. In such case, successful viral rescue and viral titers can be evaluated following the previously described protocol, without the need of primary or secondary antibodies, under a fluorescent microscope (GFP expression) [91, 96, 97, 100, 101]. Alternatively, a luciferase assay can be used to evaluate the presence of virus from TCS [91, 96, 97, 100, 101]. If the r3 arenavirus does not encode for a reporter gene, presence and quantification of the rescued r3 arenavirus should be evaluated similar to that previously described for WT arenaviruses.

3.5.3 Single-Cycle Arenavirus—As with r3 arenavirus expressing reporter genes, single-cycle infectious recombinant arenavirus expressing GFP or luciferase can be evaluated and titrated under a fluorescent microscope or using a luciferase assay, respectively [99, 102]. If the single-cycle arenavirus expresses a foreign gene that does not encode for a reporter gene, presence and titration of the virus should be evaluated by immunofluorescence, as previously described for WT arenaviruses.

3.6 Amplification of Viral Rescue

Successful arenavirus rescue depends on multiple factors, including the proper maintenance of cells used for generation of recombinant viruses, the quality of the plasmid preparations to generate recombinant arenaviruses, and the transfection efficiency of the cell line used for generation of recombinant viruses, among others. Thus, we recommend performing the arenavirus rescues in triplicate in order to increase the likelihood of a successful rescue. It is possible that arenavirus titers in TCS from initial virus rescue are low and, therefore, the viruses need to be amplified to generate a stock. To that end, we recommend infecting fresh Vero or BHK-21 cells at low MOI (0.01) and allow virus amplification for 48–72 h before collecting the new TCS for viral titration, as described above.

4 Notes

The development of arenavirus reverse genetics systems has provided investigators with a novel and powerful experimental approach to study basic aspects of arenavirus biology including the identification of viral determinants, and their mechanisms of action, which contribute to arenaviral human diseases. Moreover, the ability to manipulate the genome of

³⁰For LCMV, we use a secondary FITC-conjugated α-mouse antibody from Dako at 1:100 dilution [56, 69, 70, 91, 92, 96–98].

the prototypic arenavirus LCMV, which has proven to be a superb model system to study virus-host interactions and associated disease, and generate rLCMV with predetermined mutations, is allowing investigators to gain a detailed understanding of virus-host interactions that can lead to a large array of phenotypic outcomes of virus infection ranging from an acute and fatal meningitis to immunosuppression and chronic infections that although clinically silent can be associated with neurobehavioral abnormalities. Likewise, the generation of recombinant arenaviruses expressing appropriate reporter genes together with the development of specific cell-based assays for each of the different steps of the arenavirus life cycle will facilitate novel approaches to discover and characterize antiviral drugs against these important human pathogens. Moreover, as some of these assays do not involve the use of live forms of the virus, they overcome the difficulties posed by the need of BSL-4 facilities to handle live HF arenaviruses like LASV and JUNV.

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Abbreviations

αDG	Alpha-dystroglycan
AHF	Argentine hemorrhagic fever
ATCC	American Type Culture Collection
BHK-21	Baby hamster kidney cells
BSA	Albumin bovine serum
BSL	Biosafety level
CAT	Chloramphenicol acetyltransferase
CD	Codon optimization
CHPV	Chapare virus
Cluc	Cypridina luciferase
CPE	Cytopathic effect
cRNA	Complementary RNA
DMEM	Dulbecco's modified Eagle's medium
DN	Dominant negative
FBS	Fetal bovine serum
FDA	Food and Drug Administration

FFU	Fluorescence forming units
Gluc	Gaussia luciferase
GFP	Green fluorescent protein
GOI	Gene of interest
GP	Glycoprotein
GTOV	Guanarito virus
HF	Hemorrhagic fever
HTS	High-throughput screening
IGR	Intergenic region
ING	Investigational new drug
JUNV	Junin virus
IMP	Inosine-5'-monophosphate
L	Large RNA segment
LASV	Lassa virus
LCMV	Lymphocytic choriomeningitis virus
LF	Lassa fever
LUJV	Lujo virus
LPF2000	Lipofectamine 2000
MACV	Machupo virus
MG	Minigenome
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MOPV	Mopeia virus
mRNA	Messenger RNA
MVB	Multivesicular endosomes
NHP	Nonhuman primates
NW	New World
NP	Nucleoprotein
NS	Negative-stranded

OCEV	Ocozocoautla de Espinosa virus
ON	Overnight
ORF	Open reading frame
OW	Old World
PBS	Phosphate-buffered saline
pA	Polyadenylation signal
PICV	Pichinde virus
Pol-I	Polymerase I
Pol-II	Polymerase II
PS	Penicillin/streptomycin
RdRp	RNA-dependent RNA polymerase
Rib	Ribavirin
RT	Room temperature
r3	Recombinant tri-segmented
S	Small RNA segment
SABV	Sabia virus
S1P	Site-1 protease
SAH	S-adenosylhomocysteine
siRNA	Small interfering RNA
TCRV	Tacaribe virus
TCS	Tissue culture supernatants
TNF	Tumor necrosis factor
UTR	Untranslated regions
vRNA	Viral RNA
VSV	Vesicular stomatitis virus
vRNPs	Viral ribonucleoproteins
WWAV	Whitewater Arroyo virus
WT	Wild-type
Z	Matrix-like small RING finger protein

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Fig. 1.

Arenavirus genome organization and virion structure. **(a)** Genome organization: Arenaviruses are enveloped viruses with a single-stranded, bi-segmented RNA genome of negative polarity. Each of the two viral RNA genome segments uses an ambisense coding strategy to direct the synthesis of two viral polypeptides in opposite orientation. The small (S) RNA segment (3.5 kb, *top*) encodes the viral glycoprotein precursor (GP, *green*) and nucleoprotein (NP, *red*). The large (L) RNA segment (7.2 kb, *bottom*) encodes the RNA-dependent RNA polymerase (L, *blue*) and the small RING finger protein (Z, *orange*). **(b)** Virion structure: Arenaviruses are surrounded by a lipid bilayer containing the posttranslationally processed viral glycoprotein involved in receptor binding (GP1) and viral cell entry (GP2). Underneath the lipid bilayer is a protein layer composed of the Z protein (*orange*), which plays a major role in viral assembly and budding, and is the arenavirus counterpart of the matrix protein present in other enveloped NS RNA viruses. The core of the virus is made of a viral ribonucleoprotein (vRNP) complex, composed of the viral genome segments encapsidated by the viral NP (*red*). Incorporation of the vRNPs into newly nascent virions is mediated by NP-Z interaction. Associated with the vRNPs is the L polymerase protein (*blue*) that, together with NP, are the minimal components for viral genome replication and gene transcription. *Indicated* are also host cell ribosomes incorporated into the virus particle

Fig. 2.

Arenavirus genome replication and gene transcription: Arenavirus replication cycle takes place entirely in the cytoplasm of infected cells. The L polymerase associated with the vRNPs initiates transcription from the viral promoter located within the untranslated region (UTR, *black boxes*) at the 3' termini of the vRNAs. Primary transcription results in the synthesis of NP (**a**) and L (**b**) mRNAs from the S and L segments, respectively. Transcription termination is mediated by a secondary stem-loop structure formed by the intergenic region (IGR) found in both vRNA segments between each of the two viral genes. Subsequently, the virus polymerase L adopts a replicase mode and moves across the IGR to generate a copy of the full-length antigenome S and L vRNAs. The antigenomic RNA S and L segments serve as templates for the synthesis of GP (**a**, S segment) and Z (**b**, L segment) mRNAs. The antigenomic RNA S and L segments also serve as templates for the amplification of the corresponding viral RNA genome species. For more details *see text*

Fig. 3.

Arenavirus reverse genetics plasmids: Schematic representation of the plasmids used for the arenavirus minigenome (MG) assays, and for the generation of recombinant WT and r3 arenaviruses. **(a)** Schematic representation of the mammalian protein pCAGGS expression plasmid: Protein expression pCAGGS plasmids use the chicken β -actin promoter (*black arrow*) and the rabbit β -globin polyadenylation (pA) signal (*black box*) sequences to direct the synthesis of the viral NP (*top*) and L (*bottom*) arenavirus proteins. These plasmids are required to provide the arenavirus minimal proteins required to initiate viral gene transcription and genome replication. **(b)** Schematic representation of the polymerase I-driven vRNA expression plasmid: vRNA expression plasmid under the control of the polymerase I (Pol-I) promoter (*gray arrow*) and terminator (*gray box*) sequences directs the synthesis of the arenavirus vRNA S (*top*) and L (*bottom*) segments. For generation of recombinant arenavirus from murine or human cells, these plasmids contain the murine or human Pol-I promoters, respectively. **(c)** Schematic representation of the r3 polymerase I expression plasmids encoding the split S segment: For generation of r3 arenaviruses, the pPol-I plasmid expressing the S vRNA segment is separated into two plasmids. In the pPol-I S1 plasmid, the viral NP is replaced by GFP (*top*) and in the pPol-I S2 plasmid, the viral GP is replaced by Gluc (*bottom*). Alternatively, GFP can be expressed instead of the viral GP in the pPol-I S2 plasmid and Gluc from the pPol-I S1 plasmid. *Gray arrow* and *boxes* indicate the positions the Pol-I promoter and terminator sequences, respectively. **(d)** Schematic representation of the plasmids used for the two-plasmid arenavirus rescue system: Plasmids encoding either NP under the chicken β -actin promoter (*black arrow*) and the rabbit β -globin polyadenylation (pA) signal (*black box*) sequences, and the vRNA L segment under the Pol-I promoter (*gray arrow*) and terminator (*gray box*) sequences (pC-NP/pPol-I L), or the plasmid encoding the L protein and the vRNA S segment (pC-L/pPol-I S) are indicated. All

plasmids are ampicillin resistant (Amp^r) and contain the simian virus 40 (SV40) origin of replication. Untranslated regions (UTR, *black boxes*) and intergenic regions (IGR) in each of the vRNA segments are indicated

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Fig. 4.

Arenavirus minigenome (MG) assays: To evaluate arenavirus genome replication and gene transcription using an MG, susceptible cells (6- or 12-well plate, triplicates) are transiently co-transfected, using LPF2000, with the pCAGGS protein expression plasmids driving the expression of the viral polymerases L and NP, and the pPol-I vRNA expression plasmid S where reporter genes substitute for the viral NP (GFP) and GP (Gluc), together with a pCAGGS plasmid expressing Cluc to normalize transfection efficiencies. Recommended amounts of plasmids, LPF2000, and cells for MG assays using 12- or 6-well plates are indicated in Table 1. Reporter gene expression is then evaluated by fluorescence microscopy (GFP) using a fluorescent microscope (*bottom left*) and by Gluc activity using a luminometer (*bottom right*). Reporter gene activation (Gluc) is expressed as fold induction over cells transfected with an empty pCAGGS plasmid instead of the viral NP (negative control, -). The chicken β -actin promoter (*black arrow*) and the rabbit β -globin polyadenylation (pA) signal are indicated in the pCAGGS protein expression plasmids. Viral untranslated regions (UTR, *black boxes*) and intergenic region (IGR) in the pPol-I vRNA-like MG plasmid are indicated. The Pol-I promoter and terminator sequences in the pPol-I vRNA-like MG plasmid are indicated by a *gray arrow* and a *gray box*, respectively. For more details *see text*

Fig. 5.

Generation of recombinant wild-type (WT) arenaviruses: Arenavirus rescues are performed in rodent BHK-21 (using the mouse Pol-I promoter) or in human (using the human Pol-I promoter) cells in 6-well plate format (triplicates). Cells are transiently co-transfected, using LPF2000, with the pCAGGS protein expression plasmids encoding the viral NP and polymerase L (required to initiate viral gene transcription and genome replication) together with the pPol-I vRNA expression plasmids for the viral S and L segments (required to provide the arenavirus vRNAs to initiate viral gene transcription and genome replication). Recommended amounts of plasmids, LPF2000, and cells for LCMV WT virus rescues are indicated in Table 2a. At 72 h post-transfection, cells are trypsinized and scaled up into 10 cm dishes. After an additional 72-h incubation period, TCS are collected and presence of virus is determined by plaque assay or immunofluorescence using arenavirus-specific antibodies. The chicken β -actin promoter (*black arrow*) and the rabbit β -globin polyadenylation (pA) signal are indicated in the pCAGGS protein expression plasmids. Viral untranslated regions (UTR, *black boxes*) and intergenic region (IGR) in the pPol-I vRNA expression plasmid are indicated. The Pol-I promoter and terminator sequences in the pPol-I plasmids are indicated by *gray arrows* and *boxes*, respectively. For more details see text

Fig. 6.

Generation of recombinant WT arenaviruses using a two-plasmid-based system: A plasmid encoding NP and the vRNA L segment (pCAGGS NP/pPol-I L, *left*), along with the L polymerase protein and the vRNA S segment (pCAGGS L/pPol-I S, *right*) plasmid, is transiently co-transfected, using LPF2000, in 6-well plates (triplicates). Recommended amounts of plasmids, LPF2000, and cells for LCMV WT virus rescues using the two-plasmid-based approach are described in Table 2b. At 72 h post-transfection, cells are trypsinized and re-seeded into 10 cm dishes. After 72-h incubation, TCS are collected and presence of virus is determined by plaque assay or immunofluorescence using arenavirus-specific antibodies. The chicken β -actin promoter (*black arrow*) and the rabbit β -globin polyadenylation (pA) signal are indicated in the pCAGGS protein expression plasmids. Viral untranslated regions (UTR, *black boxes*) and intergenic region (IGR) in the pPol-I vRNA expression plasmid are indicated. The Pol-I promoter and terminator sequences in the pPol-I vRNA plasmids are indicated by *gray arrows* and *boxes*, respectively. For more details see text

Fig. 7.

Generation of recombinant tri-segmented (r3) arenavirus: Generation of r3 arenavirus is performed similarly to the rescue of recombinant WT arenavirus (Fig. 5) with the exception that the vRNA expression plasmid for the S segment (pPol-I S) is separated into two (pPol-I S1 and pPol-I S2) plasmids. In the pPol-I S1 plasmid the viral NP is replaced by GFP, and in the pPol-I S2 plasmid Gluc replaces the viral GP. Alternatively, the viral NP can be replaced by Gluc and the viral GP by GFP. Recommended amounts of plasmids, LPF2000, and cells for r3 arenavirus rescues are shown in Table 3. The chicken β -actin promoter (*black arrow*) and the rabbit β -globin polyadenylation (pA) signal are indicated in the pCAGGS protein expression plasmids. Viral untranslated regions (UTR, *black boxes*) and intergenic region (IGR) in the pPol-I vRNA expression plasmid are indicated. The Pol-I promoter and terminator sequences in the pPol-I vRNA plasmids are indicated by *gray arrows* and *boxes*, respectively. For more details *see text*

Fig. 8.

Generation of single-cycle infectious recombinant arenavirus (rLCMV GP/GFP). **(a)** Schematic representation of the pPol-I GP/GFP plasmid: LCMV GP is substituted with GFP within the pPol-I S vRNA plasmid (pPol-I GP/GFP). The Pol-I promoter (gray arrow) and terminator (gray box) sequences are indicated. Viral untranslated regions (UTR, black boxes) and intergenic region (IGR) in the pPol-I GP/GFP plasmid are indicated. **(b)** Schematic representation of the single-cycle infectious rLCMV GP/GFP rescue: BHK-21 cells (6-well plates, triplicates) are transiently co-transfected, using LPF2000, with the pCAGGS protein expression plasmids encoding NP, L, and GP (to facilitate initial viral rescue) together with the pPol-I vRNA-expressing plasmids encoding for the L and modified S (GP/GFP) segments. Recommended amounts of plasmids, LPF2000, and cells for single-cycle arenavirus rescues are indicated in Table 4. At 72 h post-transfection, TCS are scaled up. After additional 72-h incubation, TCS are collected and used to infect BHK-21 cells constitutively expressing LCMV GP and virus rescue is confirmed by the appearance of GFP loci. The chicken β -actin promoter (black arrow) and the rabbit β -globin polyadenylation (pA) signal are indicated in the pCAGGS protein expression plasmids. Viral untranslated regions (UTR, black boxes) and intergenic region (IGR) in the pPol-I vRNA expression plasmid are indicated. The Pol-I promoter and terminator sequences in the pPol-I vRNA plasmids are indicated by gray arrows and boxes, respectively. **(c)** Generation of GP-pseudotyped single-cycle infectious rLCMV GP/GFP: Single-cycle infectious rLCMV GP/GFP is used to infect BHK-21 cells constitutively expressing LCMV (left, gray) or LASV (right, red) GP to produce GP-pseudotyped LCMV (pLCMV) and LASV (pLASV). For more details see text

Table 1

Plasmid DNA and LPF2000 concentrations for arenavirus minigenome (MG) assays: recommended amounts of pCAGGS and pPol-I plasmids and LPF2000 required to assess viral gene replication and genome transcription using an arenavirus MG assay in 12-well (*left*, $5.0\text{--}6.0 \times 10^5$ cells/well) or 6-well (*right*, $1.0\text{--}1.2 \times 10^6$ cells/well) plates are indicated

Plasmid	12-Well plates (μg)	6-Well plates (μg)
pCAGGS NP	0.3	0.6
pCAGGS L	0.6	1.2
pPol-I S MG	0.5	1.0
pCAGGS Cluc	0.1	0.2
Total DNA	1.5	3.0
LPF2000	3.75	7.5
Cells	$\sim 5.0\text{--}6.0 \times 10^5$ /well	$\sim 1.0\text{--}1.2 \times 10^6$ /well

Table 2

LPF2000/plasmid DNA concentrations for arenavirus rescues: recommended amounts of pCAGGS and pPol-I plasmids and LPF2000 to generate recombinant WT arenaviruses using the 4 (A) and the 2 (B) plasmid approaches in 6-well ($1.0\text{--}1.2 \times 10^6$ cells/well) plates are indicated

Plasmid	6-Well plates (μg)
(A)	
pCAGGS NP	0.8
pCAGGS L	1.0
pPol-I S	0.8
pPol-I L	1.4
Total DNA	4.0
LPF2000	10
Cells	$\sim 1.0\text{--}1.2 \times 10^6/\text{well}$
(B)	
pCAGGS NP/pPol-I L	2.0
pCAGGS L/pPol-I S	1.0
Total DNA	3.0
LPF2000	7.5
Cells	$\sim 1.0\text{--}1.2 \times 10^6/\text{well}$

Table 3

Plasmid DNA and LPF2000 conditions to generate recombinant tri-segmented (r3) arenaviruses: Amounts of plasmid DNA and LPF2000 recommended to generate r3 arenaviruses using 6-well ($1.0\text{--}1.2 \times 10^6$ cells/well) plates are indicated

Plasmid	6-Well plates (μg)
pCAGGS NP	0.8
pCAGGS L	1.0
pPol-I S1	0.8
pPol-I S2	0.8
pPol-I L	1.4
Total DNA	4.8
LPF2000	12
Cells	$\sim 1.0\text{--}1.2 \times 10^6$ /well

Table 4

Amount of plasmid DNA and LPF2000 to generate recombinant single-cycle infectious LCMV: Plasmid DNA and LPF2000 concentrations are indicated to generate a recombinant single-cycle infectious LCMV using 6-well ($1.0\text{--}1.2 \times 10^6$ cells/well) plates

Plasmid	6-Well plates (μg)
pCAGGS NP	0.8
pCAGGS L	1.0
pCAGGS GP	0.8
pPol-I GP/GFP	1.0
pPol-I L	1.4
Total DNA	5
LPF2000	12.5
Cells	$\sim 1.0\text{--}1.2 \times 10^6$ /well