Preparation of Recombinant Alphaviruses for Functional Studies of ADP-Ribosylation

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Abstract

Recently we characterized the mono(ADP-ribosyl) hydrolase (MAR hydrolase) activity of the macrodomain of nonstructural protein 3 (nsP3MD) of chikungunya virus. Using recombinant viruses with targeted mutations in the macrodomain, we demonstrated that hydrolase function is important for viral replication in cultured neuronal cells and for neurovirulence in mice. Here, we describe the general cell culture and animal model infection protocols for alphaviruses and the technical details for biochemical characterization of the MAR hydrolase activity of nsP3MD mutants and the preparation of recombinant viruses incorporating those mutations through site-directed mutagenesis of an infectious cDNA virus clone.

Key words Alphavirus, Chikungunya virus, Sindbis virus, MAR hydrolase assay, Infectious cDNA clone, Reverse genetics, Site-directed mutagenesis, In vitro transcription

1 Introduction

Alphaviruses are mosquito-borne plus-strand, enveloped RNA viruses that cause a variety of human diseases ranging from rash and arthritis by the “Old World” viruses to encephalitis by the “New World” viruses. The encephalitic alphaviruses, like Venezuelan, western, and eastern equine encephalitis viruses, are endemic in the Americas, and recently introduced arthritic alphaviruses like chikungunya virus (CHIKV) can also cause atypical complications like encephalitis [1, 2]. Alphaviruses have an approximately 11.7 kilobase (kb) positive-sense RNA genome with a 5′ 7-methylguanosine cap and 3′ poly-A tail that encodes four nonstructural proteins (nsP1–4) and six structural proteins (C, E3, E2, 6K/TF, and E1) that are expressed from a subgenomic RNA [3]. Recently, we have shown that the macrodomain of viral nonstructural protein 3

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(nsP3) of CHIKV enzymatically reverses the protein modification mono(ADP-ribosyl)ation in vitro. Macrodens are a conserved protein fold found from archaea to higher eukaryotes and are characterized by their binding to the small molecule ADP-ribose and its derivatives [4]. The viral macrodomain is part of the nonstructural protein in a subset of positive-strand RNA viruses, such as alphaviruses, coronaviruses, and hepatitis E virus [5, 6]. We identified and mutated residues in nsP3 critical for mono(ADP-ribosyl) binding and hydrolase (MAR hydrolase) activity, and using mutants with decreased MAR hydrolase activity, we demonstrated that reduction of this activity impairs viral replication in cultured mammalian and mosquito cells and virulence in neonatal mice [7].

Reverse genetic approaches have been extensively used in alphavirus research to understand virus biology and replication [8, 9], vector competence [10–13], attenuation and virulence [14, 15], and immunogenicity [16, 17]. The approach involves the construction of an infectious cDNA clone by incorporating a DNA copy of the viral RNA genome into a plasmid vector. With incorporation of the bacteriophage T7 or SP6 RNA polymerase promoters, the cDNA can be in vitro transcribed to produce full-length viral RNA transcripts. Transfection of the transcribed RNA into permissive cells results in production of infectious virus. To understand a loss or gain of gene function, variants or mutants are made in the infectious cDNA clone to produce mutant viruses in the same virus background for analysis.

In our studies of the MAR binding and hydrolase activities of the nsP3 macrodomain, we introduced mutations into the macrodomain region of nsP3 in the infectious cDNA clone of the 181/clone 25, the live attenuated vaccine strain of CHIKV. The 181/25 virus was derived from the Southeast Asian strain AF15561 by 18 plaque-to-plaque passages in MRC-5 human lung fibroblasts [18]. The infectious clone of 181/25 was prepared by employing site-directed mutagenesis to introduce five non-synonymous mutations into the parent clone 15,561 [10]. The parent clone 15,561 in a modified pSinRep5 plasmid was engineered to possess a SP6 RNA polymerase promoter at the 5’ terminus and a poly(A) tail, followed by a NotI restriction site at the 3’terminus [15] (Fig. 1). Because 181/25 is a vaccine strain, we reversed the attenuating mutations I12T and R82G [15] in the E2 glycoprotein to generate a recombinant CHIKV strain (181/25E2I12T/R82G) that is virulent for newborn mice.

In this chapter, we describe the technical details of our strategies for biochemically characterizing the nsP3MD mutants with MAR hydrolase assays and for incorporating the mutations into the viral genome by site-directed mutagenesis. Here, the general protocols for cell culture of alphaviruses, infection of mice, and generation of recombinant mutant viruses using chikungunya virus 181/25 infectious cDNA clone are explained. Variations in the methodology for other alphaviruses, particularly the prototype Sindbis virus, are also mentioned. A summary of the methodology is outlined in Fig. 2.
2 Materials

2.1 MAR Hydrolase Assay

1. Recombinant human PARP10 catalytic domain 818–1025 (PARP10CD).
2. Recombinant MAR hydrolases (e.g., chikungunya viral macrodomain and human MacroD2).
3. $^{32}$P-labeled $\beta$-nicotinamide adenine dinucleotide ($^{32}$P-NAD$^+$) (PerkinElmer).
4. Micro Bio-Spin 6 buffer exchange column.
5. Geiger counter.
6. Invitrogen Novex 14% Tris-glycine gel 10-well.
7. Automodification (AM) buffer: 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 20 mM beta-mercaptoethanol, 5 mM MgCl$_2$.
8. Demodification (DM) buffer: 25 mM Tris-HCl pH 7.5, 200 mM NaCl, 20 mM beta-mercaptoethanol.
9. 4× SDS-PAGE sample loading buffer.
10. Novex SimplyBlue Safe Stain.
11. FujiFilm autoradiograph imaging plate.
12. FujiFilm imager.

2.2 Site-Directed Mutagenesis of cDNA and Transformation of Competent Bacteria

1. 10 ng/μL of full-length cDNA clone (e.g., CHIKV 181/25 cDNA clone).
2. QuikChange II site-directed mutagenesis kit.
3. 20 U DpnI.
4. One shot max efficiency chemically competent DH5α E. coli cells.
5. SOC medium: 2% tryptone (w/v), 1% yeast extract (w/v), 8.5 mM NaCl, 20 mM glucose, 2.5 mM KCl, 10 mM MgCl₂.
6. LB agar plates with ampicillin: 10% Bacto tryptone (w/v), 5% yeast extract (w/v), 10% NaCl (w/v), 2% Bacto agar (w/v), 100 μg/mL ampicillin.
7. LB broth with ampicillin: 10% Bacto tryptone (w/v), 5% yeast extract (w/v), 10% NaCl (w/v), 100 μg/mL ampicillin.
8. QIAprep spin miniprep kit.

2.3 Linearizing the Mutant Plasmid and In Vitro RNA Transcription

1. Restriction digestion buffer, 30 U NotI.
2. Purification of linearized plasmid: phenol-chloroform mix, chloroform, 3 M sodium acetate, 95% ethanol, nuclease-free water.
3. mMESSAGE mMACHINE SP6 transcription kit.
4. Purification of in vitro transcribed RNA: MEGAclear™ kit.

2.4 Transfection of RNA Transcripts into Susceptible Cells

1. BHK-21 cells (baby hamster kidney epithelial cells).
2. Growth media: Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL).
3. Maintenance media: DMEM supplemented with 1% heat-inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL).
4. 1× Dulbecco’s PBS (1.1 g Na₂HPO₄, 0.25 g KH₂PO₄, 0.2 g KCl, 8 g NaCl in IL distilled water).
5. 1× 0.5% trypsin EDTA.
6. OptiMEM serum-free media.
7. Lipofectamine 2000.

2.5 Viral RNA Extraction

1. Cell culture supernatant containing virus.
2. QIAamp® viral RNA mini kit.
3. RNaseZap.
2.6 One-Step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

1. Superscript III one-step RT-PCR system: 1× reaction mix, 2 μL Superscript™ III RT/Platinum™ Taq Mix, 40 U ribonuclease inhibitor, 10 pmol of each oligonucleotide primer (see cf. Note 11).

2.7 Agarose Gel Electrophoresis and DNA Purification

1. 1% TAE-agarose gel (100 mL): 1 g agarose in 100 mL 1× TAE buffer (4.84 g Tris base, 0.58 g EDTA, and 1.1 mL glacial acetic acid in 1 L distilled water), 10 μL ethidium bromide (stock concentration 10 mg/mL).
2. 1 kb DNA ladder.
3. UV transilluminator.
4. QIAquick® gel extraction kit.
5. QIAquick® PCR purification kit.

2.8 Quantification of Virus by Plaque Assay

1. Vero cells (African green monkey kidney epithelial cells).
2. Growth media, maintenance media, 1× Dulbecco’s PBS, 1× 0.5% trypsin EDTA.
3. 2× MEM supplemented with 1% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL).
4. 1.2% Bacto agar (w/v) in water.
5. 10% formaldehyde in PBS.
6. 1% crystal violet (w/v) in 20% ethanol in water.

2.9 Infection of Neuronal Cells

1. NSC34 cells (mouse neuronal cells) [19].
2. Growth media, maintenance media, 1× Dulbecco’s PBS, 1× 0.5% trypsin EDTA.
3. RNeasy plus mini kit.
4. RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) NP40, 0.5% (w/v) sodium orthovanadate, 1 mM EDTA pH 8), 1× protease inhibitor cocktail.

2.10 Infection of Newborn Mice

1. Timed pregnant CD-1 mice.
2. Inoculation of mice-29 G needle, anesthesia chamber, isoflurane.
3. Serum collection-1 mL 25 G needle, microtainer.
4. Ice cold PBS, 20 mL syringe, butterfly needle, dry ice.

2.11 Antiviral IgG and IgM Enzyme Immunoassay

1. 96-well flat immunoplates MaxiSorp.
2. 50 mM bicarbonate coating buffer pH 9.6 (1.58 g Na₂CO₃ and 2.92 g NaHCO₃ in 1 L distilled water).
3. 1× Dulbecco’s PBS (1.1 g Na₂HPO₄, 0.25 g KH₂PO₄, 0.2 g KCl, 8 g NaCl in 1 L distilled water).
3 Methods

3.1 MAR Hydrolase Assay

MAR hydrolase assay involves automodifying the substrate PARP10\(^{CD}\) with radiolabeled \(^{32}\text{P}-\text{NAD}^+\) and incubating the substrate with recombinant proteins to be tested for MAR hydrolase activity followed by SDS electrophoresis and phosphoimaging.

1. Automodify desired amount of PARP10\(^{CD}\) (*see Note 1*) with \(^{32}\text{P}-\text{NAD}^+\) at a concentration of 0.25 \(\mu\text{Ci}/\mu\text{g}\) of PARP10\(^{CD}\) in AM buffer at 30 °C for 30 min.

2. Add 1 mL of DM buffer to Bio-Rad Micro Bio-Spin column and allow gravity flow through the column. Repeat twice to equilibrate column in DM buffer. Add PARP10\(^{CD}\) automodification reaction to the top of the bed (100–150 \(\mu\text{L}\) total volume) and allow it to enter the column. Add 600 \(\mu\text{L}\) of DM buffer to the column and collect 2-drop fractions using the Geiger counter to determine when the \(^{32}\text{P}-\text{labeled PARP10}^{CD}\) is eluted. Once the \(^{32}\text{P}-\text{labeled PARP10}^{CD}\) begins to elute, collect a 500 \(\mu\text{L}\) fraction to maximize yield and desalting of unincorporated \(^{32}\text{P}-\text{NAD}^+\).

3. For each reaction, make 1 \(\mu\text{g}\) aliquots of desalted \(^{32}\text{P}-\text{labeled PARP10}^{CD}\) (*see Note 2* about concentration of desalted sample from step 2). Add recombinant wild-type (WT), mutant MAR hydrolases, or DM buffer alone to reaction at concentrations equimolar to amounts of automodified PARP10\(^{CD}\) (1 \(\mu\text{g}\) of PARP10\(^{CD}\) is 43.1 pmol) and take reactions up to 15 \(\mu\text{L}\) total volume with DM buffer.

4. Incubate reactions at 37 °C for 1 h and stop reactions by adding 5 \(\mu\text{L}\) of 4× SDS-PAGE sample loading buffer.

5. Run samples by SDS-PAGE on Invitrogen Novex 14% Tris-glycine gel 10-well.

6. Rinse gel for 10 min with Milli-Q water followed by staining with Novex SimplyBlue Safe Stain for 1 h at room temperature. Once bands develop, rinse gel in Milli-Q water for 1 h at room temperature.
7. Image gel with a scanner to obtain total protein stain and expose the gel to a FujiFilm autoradiograph imaging plate overnight.

8. Image FujiFilm autoradiograph imaging plate on FujiFilm imager to acquire autoradiograph of gel.

9. For quantification, open the autoradiography file in ImageJ and quantify the signal intensity of each lane using the GelAnalyzer tool (see Note 3). Quantify the intensity of the $^{32}$P signal from automodified PARP10$^{cp}$ and obtain the percentage of signal removed using the below equation:

$$\%^{32}P - \text{signal removed} = \left( \frac{\text{Intensity from buffer alone} - \text{Intensity from sample}}{\text{Intensity from buffer alone}} \right) \times 100\%$$

To obtain % WT activity, use the below equation in combination with the above equation:

$$\%\text{WT activity} = \left( \frac{\left(\%^{32}P - \text{signal removed from mutant MAR hydrolase}\right)}{\left(\%^{32}P - \text{signal removed from WT MAR hydrolase}\right)} \right) \times 100\%$$

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### 3.2 Site-Directed Mutagenesis of Infectious cDNA Clone and Transformation of Competent Cells

The infectious cDNA clone which is the double-stranded copy of the viral genome carried on a plasmid vector is modified for introducing mutation by PCR-based site-directed approach, followed by transformation to the E. coli cells. The mutant clone is then subjected to sequencing for checking the presence or absence of mutation.

1. Design oligonucleotide forward and reverse primers containing the desired mutation (see Note 4).

2. Prepare each PCR reaction in a 50 μL reaction volume:
   - 10× buffer 5 μL
   - *Pfu* turbo polymerase (2.5 U/μL) 1 μL
   - dNTP mix (10 mM) 1 μL
   - Template plasmid (10 ng) 1 μL
   - Forward primer (10 μM) 1.25 μL
   - Reverse primer (10 μM) 1.25 μL
   - Nuclease-free water Make up to 50 μL

3. Quickly spin the PCR tubes in a Mini Microcentrifuge and load them into a PCR thermal cycler. The thermal cycling conditions are 1 min of initial denaturation at 95 °C, followed by 18 cycles of the following: denaturation at 95 °C for 50 s, annealing at 60 °C for 15 s, extension at 68 °C for 16 min, and
a final extension at 68 °C for 7 min. After the reaction, cool the tubes on ice for 2 min.

4. To eliminate the template plasmid, digest with DpnI which cleaves only at methylated sites. Add 1 μL of DpnI (20 U/μL) endonuclease directly to the reaction, mix by pipetting. Quickly spin the PCR tubes in a Mini Microcentrifuge, and incubate at 37 °C for 1 h in the thermal cycler.

5. Transform competent DH5α cells with 2 μL of the DpnI digested product. Flick the tubes gently to mix and incubate the tubes on ice for 30 min. Heat shock the cells at 42 °C for 30 s, and immediately incubate the tubes on ice for 2 min. Add 250 μL of SOC media, and incubate at 37 °C for 1 h with continuous shaking at 200 rpm for recovery. Plate 100 μL of the transformation mixture onto LB agar plates with ampicillin, and incubate the plates at 37 °C for 16–18 h.

6. Pick individual E.coli colonies from the plate, and inoculate in 5 mL LB broth with ampicillin and incubate 16–18 h at 37 °C with continuous shaking at 200 rpm. Pellet the bacterial cells at 3000 rpm (1942 rcf) for 10 min at room temperature, and proceed with plasmid isolation using QIAprep spin mini plasmid prep kit or any other commercial mini prep kit (see Note 5).

7. The plasmids are sequenced and compared with the sequence of the parent clone (see Note 6).

### 3.3 Linearizing the Mutant Plasmid DNA and In Vitro RNA Transcription

In order to make the mutant viruses, the mutant full-length cDNA clones are linearized, purified, and in vitro transcribed to RNA.

Linearize 3 μg of plasmid containing the desired mutation in the full-length cDNA with NotI enzyme in a 100 μL reaction (see Note 7). Incubate the reaction for 16–18 h at 37°C. The component volumes for the reaction are:

- 10× restriction buffer 10 μL
- Plasmid DNA 3 μg
- NotI (20 U/μL) 1.5 μL
- Nuclease-free water Make up to 100 μL

Analyze 5 μL of the digested reaction by gel electrophoresis to ensure that the plasmid is fully linearized.

1. Purification of the linearized plasmid (100 μL) is performed by phenol-chloroform extraction, followed by alcohol precipitation in an Eppendorf tube. Add an equal volume of phenol:chloroform to the digested product, mix well, and centrifuge at 15,700 rcf for 5 min. Transfer the aqueous phase to a new tube and add an equal volume of chloroform, mix well, and centrifuge at 15,700 rcf for 10 min. Harvest the aqueous phase and add 1/10 volume 3 M sodium acetate and 2 volumes 95% ethanol and incubate at −20 °C for 1 h. After 1 h, centrifuge at 13,000 rpm (15,700 rcf) for 15 min at 4 °C and discard super-
natant fluid completely and air-dry the tube. Resuspend the pellet in 10 μL nuclease-free water, and quantify the purified plasmid DNA with a NanoDrop spectrometer (see Note 8).

2. Transcribe 1.5 μg of purified plasmid DNA into viral RNA using mMESSAGE mMACHINE SP6 kit. The reaction mixture consists of:

- 10× reaction buffer 3 μL
- GTP (20 mM) 3 μL
- 2× NTP/CAP 10 μL
- Linear template 1.5 μg
- Enzyme mix 2 μL
- Nuclease-free water Make up to 30 μL

Mix the reaction by pipetting thoroughly and incubate at 37 °C for 2 h. Add 1 μL of DNase to digest the template DNA, mix by pipetting, and incubate further at 37 °C for 15 min.

Purify the RNA transcripts using the MEGAclear™ kit, and elute with 30 μL of RNase-free water (see Note 9). Generally, the expected concentration of the RNA synthesized from a standard 30 μL reaction starting from 1.5 μg of DNA is within a range 2–3 μg/μL.

3.4 Transfection of BHK-21 Cells to Recover Virus

This section deals with the transfection of the CHIKV RNA transcript into permissive cell lines to recover the clone-derived virus.

1. Transfer BHK-21 cells in growth medium into a T150 cm² flask, and incubate for 18–24 h in 37 °C 5% CO₂ incubator so that the cells will be 80–90% confluent at the time of transfection (see Note 10).

2. Transfect the in vitro transcribed RNA into cells using Lipofectamine 2000. Dilute 40 μL of Lipofectamine 2000 in 200 μL OptiMEM. Mix gently and keep at room temperature for 5 min. Also dilute 20–40 μg of in vitro transcribed RNA in 200 μL OptiMEM without serum and mix gently. Combine the diluted RNA with the diluted Lipofectamine (total volume is 400 μL). Mix by gentle pipetting, and incubate the mix for 20 min at room temperature to allow the RNA-Lipofectamine complex to form.

3. Remove the culture media from the T150 cm² flask of BHK-21 cells, wash once with PBS, and add 7 mL of OptiMEM without serum. Add the RNA-Lipofectamine 2000 complex drop by drop on top of the cells. Swirl gently to ensure equal distribution of the transfection mix to the cells.

4. Incubate the cells at 37 °C, 5% CO₂ for 6 h, with gentle rocking of the flask back and forth every 30 min. After 6 h, remove the transfection mix and add 12 mL DMEM 1% FBS to the flask. Incubate transfected cells at 37 °C, 5% CO₂ for 24–48 h until cytopathic effect (CPE) is visible (Fig. 3).
5. Collect the viral supernatant fluid from the flasks into a sterile 50 mL tube, and centrifuge at 1000 rpm (216 rcf) for 10 min at 4 °C. Virus should be stored as 1 mL aliquots in cryopreservation tubes at −80 °C (label as passage 0, P0).

The presence of the mutation in the clone-derived virus is verified by RNA isolation, followed by reverse transcriptase PCR, and finally sequencing.

1. Clean the work bench and pipettes with RNase Zap to remove all potential RNase contamination.

2. Extract viral RNA from viral supernatant fluid using QIAamp® viral RNA kit and store at −80 °C.

3. Perform one-step reverse transcriptase PCR using 5 μL of extracted RNA (see Note 11).

   2× reaction buffer 25 μL
   RNA template 5 μL
   Forward primer (10 μM) 1.25 μL
   Reverse primer (10 μM) 1.25 μL
   Superscript RT 2 μL
   RNaseOUT 1 μL
   Nuclease-free water Make up to 50 μL

4. Quickly spin the PCR tubes in a Mini Microcentrifuge and load them into a PCR thermal cycler. The thermal cycling conditions are 30 min of reverse transcription step at 50 °C and 2 min of initial denaturation at 94 °C, followed by 40 cycles of the following: denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, extension at 68 °C for 2 min, and a final extension at 68 °C for 5 min.
5. Perform agarose gel electrophoresis with a few microliters of the PCR product to determine whether the expected band is present. Mix 5 μL of the PCR reactions with 1 μL of loading dye, and load into individual wells. Load 1 kb DNA ladder into one of the wells to identify the expected band size. Run the agarose gel electrophoresis at 100 V for about 45 min or until the loading dye is nearing the bottom edge of the gel. Transfer the gel into a UV transilluminator to identify the expected band.

6. Purify the PCR product to remove excess primers and other PCR reaction mix components. If there is only a single band present, purify the remaining PCR product directly using QIAquick® PCR purification kit. If there are additional bands, excise the correct band from the gel and purify using QIAquick® gel extraction kit.

7. The purified PCR product will be used as a template for sequencing to confirm the presence or absence of the mutation in the clone-derived virus. The purified PCR product can be stored at −20 °C (see Note 6 for the primer details).

1. Culture BHK-21 cells in growth medium in a T150 cm² flask the day before infection so that they are 80–90% confluent at the time of infection.

2. Infect the cells with a 1:10 dilution of P₀ virus (i.e., virus obtained after in vitro transcription; see Subheading 3.4, step 5). Incubate the cells for 1 h at 37 °C, with gentle rocking of the flask back and forth every 15 min. After 1 h, remove the unadsorbed virus, wash once with PBS, and add a fresh 12 mL of DMEM 1% FBS. Incubate the cells for 24–48 h until cytopathic effect is visible.

3. Collect supernatant containing the virus from the flasks into a sterile 50 mL tube, and centrifuge at 216 rcf for 10 min at 4 °C. Virus should be stored as 1 mL aliquots in cryopreservation tubes at −80 °C (label as passage 1, P₁).

4. Characterize the virus (P₁) by sequencing to confirm the presence of the desired mutation (follow Subheading 3.5), and proceed for virus quantitation by plaque assay titration.

Detecting the viral load or viral titer is essential for any virology-based study. Plaque assay allows the quantification of infectious virus particles which rely on the ability of viruses to cause cytopathic effects on the host cells because of infection. This section describes the steps involved: infection of cell monolayers with tenfold dilutions of viral supernatant, followed by overlaying with media having high molecular weight substrate like Bacto agar, agarose, or carboxymethylcellulose, which will limit the viral spread.
The plaques thus formed are visualized by staining cell monolayers with crystal violet or neutral red.

1. Day 1: Plate Vero cells in DMEM 10% FBS in a 6-well plate. A 100% confluent flask of Vero cells can be plated into five 6-well plates. Each plate will be for one sample; each well within this plate is for a tenfold dilution of that sample (Fig. 4). Incubate the plates overnight at 37 °C, 5% CO₂ (see Note 13).

2. Day 2: Infection

(a) Preparation of overlay agar mixture – Melt 1.2% Bacto agar in the microwave and place in 42 °C water bath. Warm 2× MEM 1% FBS in 42 °C water bath. Mix equal amounts of 1.2% Bacto agar and 2× MEM and place in 42 °C water bath until use (see Note 14).

(b) Sample preparation – Prepare tenfold serial dilutions of samples in 48-well plates.

Add 450 μL of DMEM 1% FBS in each well of 48-well plate. Add 50 μL of viral stocks to be titrated to the wells in first row, mix well and transfer 50 μL from first row to second row, and thus continue serial dilutions as shown in Fig. 5.

(c) Infection of Vero cells – Aspirate the media from the wells and wash briefly with PBS. Add 200 μL of serially diluted samples to the appropriate wells. Incubate at 37 °C in 5% CO₂ incubator for 1 h with gentle shaking every 15 min. After 1 h of incubation, aspirate the virus, wash with PBS, and add 1.5 mL overlay agar mixture to each well. Let agar solidify at room temperature, and incubate the plates at 37 °C, 5% CO₂, for 48 h.

![Fig. 4 Plate map of 6-well plate for the plaque assay](image-url)
3. Day 4: Plaque staining and counting – Fix the cells by adding 1 mL of 10% formalin in PBS directly into the wells without removing the agar overlay. Incubate for 2 h to overnight (preferred). Aspirate the fixed overlay agar to an appropriate waste container, invert and tap the plate on a tissue paper pad to remove the agar overlay, and stain by adding 300 μL of 1% crystal violet in 20% ethanol in water. Keep for 10 min, and after cells are stained, wash in running tap water to remove excess stain solution, and air-dry the plates for an hour (Figs. 6 and 7).

Calculate the PFU/mL = \frac{(\text{plaque count}) \times (\text{dilution factor})}{(\text{amount of sample added in mL})}

In vitro infection in cells involves plating cells, calculating the amount of the infectious virus to be added, infection of the cells, followed by incubation for the respective time points.

1. Seed $1 \times 10^5$ NSC34 cells per well in growth medium into 6-well plates a day before so the cells will be 80–90% confluent at the time of infection. Include one well for cell counts to calculate the multiplicity of infection (MOI). MOI is the ratio of plaque-forming units (PFU) to the total number of cells in the well. If one million virions are added to one million cells, the MOI is one.

2. Next day, trypsinize one of the wells in the plate, count the cells, and calculate the amount of virus needed for the desired
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MOI. Prepare the diluted virus stock in DMEM 1% FBS for one experiment (if it involves two or more wells) in one tube to ensure homogeneity.

3. Remove the media from the cells, wash with PBS, and add 200 μL of diluted virus to the corresponding wells for infection. Add media alone to the mock infected wells. Incubate for 1 h with gentle swirling every 15 min at 37 °C, 5% CO₂. After 1 h of incubation, remove the unadsorbed virus, wash once with 1 mL PBS, and add 1.5 mL of DMEM 1% FBS.
4. Collect the supernatant at the desired time points and freeze at 
−80 °C. The supernatants can be further assayed for infectious 
virus titers using a plaque assay (follow Subheading 3.7) or for 
levels of secreted proteins such as cytokines by ELISA. The 
cells from each well can be collected either in buffer RLT (from 
Qiagen kit) for RNA isolation or in RIPA buffer for protein 
studies.

**3.9 Infection of Mice**

**to Assess**

**Neurovirulence**

**and Virus Growth (See**

**Note 15)**

Studies using mouse models to evaluate neurovirulence can be car-
rried out by infecting the mice intracranially and monitoring them 
daily for clinical signs/mortality. The organs can be harvested to 
assess the viral replication by plaque assay or RNA quantification.

1. All animal experiments and protocols must be performed 
according to the guidelines approved by the Institutional 
Animal Care and Use Committee and carried out with strict 
adherence to the Guide for the Care and Use of Laboratory 
Animals by the National Institutes of Health and the Policy on 
Humane Care and Use of Laboratory Animals by the US 
Public Health Service.

2. The CHIKV mutant viruses in the virulent background 
181/25E2 I12TR82G must be handled in the Biosafety level three 
facility and the animal experiments in the ABSL3 facility 
according to US regulations. All experiments with the biologi-
cal samples after infection with CHIKV in the virulent back-
ground 181/25E2 I12TR82G must be handled in the BSL3 
facility.

3. The mice should be bred under barrier conditions with a stan-
dard 12-h light/12-h dark cycle.

4. Timed pregnant CD-1 female mice are maintained in an 
ABSL3 facility, and the CHIKV infection experiments are con-
ducted in 2-day old neonates.

5. The route of administration is intracranial in a 10 μL volume 
having 1000 PFU of virus diluted in PBS. Prepare the virus 
dilution for one experiment (if it involves more animals for 
same virus) in one tube to ensure homogeneity, and keep on 
ice until use to prevent thermal inactivation. The same mutant 
virus is administered to all pups of a litter.

6. Anesthetize each pup prior to infection using isoflurane (see 
Note 16).

7. Restrain the pup manually on a solid surface by firmly placing 
your thumb and index finger on either side of the head just at 
the nape, and insert the needle having 10 μL of virus into the 
right side of the cranium approximately halfway between the 
eye and the ear just off-center from the midline (see Note 17).

8. Monitor the mice twice daily for weight gain/loss and mor-
bidity/mortality. If any animal shows signs of being moribund
or reaches experimental end points, euthanize immediately according to a method which has been approved by the ethics governing body. The 2-day old CD-1 mice infected with virulent strains of CHIKV will die without showing much evidence of illness, so clinical scoring is not possible for this model (see Note 18).

9. At the end of the morbidity/mortality experiment, the surviving pups and the mother are generally sedated with isoflurane and euthanized according to a method which has been approved by the ethics governing body.

10. For time course infection experiments to assess virus replication, at least three pups per group are euthanized at each time after infection, and blood and organs are collected. The pups are anesthetized using isoflurane, and blood is collected with a 1 mL syringe by cardiac puncture. The blood can be transferred to a microtainer tube and spun at 1500 rpm (200 rcf) for 15 min to collect serum.

11. Expose the heart, and perfuse with 20 mL ice cold PBS to remove the blood from the tissues before collecting the organs. When collecting the brain, one half brain can be homogenized in 1 mL QIAzol lysis reagent for RNA isolation using RNeasy lipid tissue mini kit. Genetic characterization of virus replicating in the brain is carried out by sequencing (see Subheading 3.5). The other half of the brain can be homogenized in PBS (see Note 19) and used for assessing the infectious virus titer using plaque assay (see Subheading 3.7). The viral titer is calculated using the formula:

\[
\text{PFU/g} = \left( \frac{(\text{plaque count}) \times \text{dilution factor}}{(\text{amount sample added in mL})} \right) \times \text{homogenate percent}
\]

3.10 Measurement of IgG and IgM Antibody Responses of Infected Mice

Levels of viral-specific IgM are quantified to evaluate the immediate, early humoral response following infection, while levels of viral-specific IgG are quantified to define the more specific humoral response that occurs later following infection and following reexposure. The section describes measuring and quantifying them through ELISA.

1. To coat plates, add 50 μL of coating buffer containing 10⁶ PFU of virus to each well of a 96-well immunoplate (gently tap the plate against the bench to make sure the coating solution completely covers the entire well surface). Seal with saranwrap and incubate at 4 °C overnight.
2. Wash plate once with washing buffer, and add 200 μL of blocking buffer containing FBS. Incubate at 37 °C for 2 h or 4 °C overnight.

3. Wash plate twice with washing buffer, tap against paper towel to remove excess solution, and add 50 μL of sample in blocking buffer into each well. Cover with saranwrap and incubate at 4 °C overnight. The serum can be diluted 1:100 and brain homogenates 1:2 for 10% or 1:4 for 20% homogenates. Day 0 serum sample or blocking buffer alone must be included as a negative control.

4. Wash plate four times with washing buffer, and add 50 μL of secondary antibody (goat anti-mouse IgG-HRP/goat anti-mouse IgM-HRP) in blocking buffer in a dilution 1:1000 at room temperature for 2 h.

5. Wash plate four times with washing buffer, and add 100 μL of BD OptEIA TMB substrate reagent kit (see Note 20).

6. Stop the reaction by adding 50 μL stop solution to each well. A yellow color will develop, and read the optical density at 450 nm immediately.

### Notes

1. We used 1 μg of automodified PARP10CD per reaction but performed the automodification in bulk up to 100 μg. The automodification and desalting procedure can be applied to other MARylated substrates.

2. Desalting on the Bio-Rad Micro Bio-Spin 6 column causes sample dilution and loss. We automodified 85 μg of PARP10CD in 100 μL for step 1 and recovered 50 μg of 32P-labeled PARP10CD in 500 μL after step 2. Concentration can be empirically determined by SDS-PAGE and coomassie blue staining with standards of known concentrations.

3. Extensive tutorials of this tool for quantifying band signals can be found online (e.g., [http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/](http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/)).

4. Design the oligonucleotide primers as follows. The primers should be between 25 and 45 bases in length, with a melting temperature (Tm) of ≥78 °C. The desired mutation should be in the middle of the primer with ~10–15 bases of correct sequence on both sides. Adapted from quick change site-directed manual [https://www.agilent.com/cs/library/user-manuals/Public/200523.pdf](https://www.agilent.com/cs/library/user-manuals/Public/200523.pdf).
5. Glycerol stock for the positive clones can be prepared and stored. But it is advisable to retransform the plasmid DNA and grow a bacterial culture from freshly grown culture to obtain a higher yield of plasmid DNA.

6. For sequencing the macrodomain region of CHIKV, CHK nsp3 MDR primer 5’-GGTACCCTCGAGTCATTAGG TCCGCATCTGTATGGCCTC-3’ was used. For sequencing the larger plasmid, at least 100 ng/μL is required. The sequencing primer anneals to the positive strand of the virus.

7. The restriction digestion enzyme for linearizing the plasmid is chosen depending on the restriction site engineered into the cDNA clone. For the 181/25 cDNA clone, Not I site is included at the 3’terminus (see Fig. 1).

8. For purification of the plasmid DNA, commercial PCR purification kits can be used. But for larger plasmids, the final yield will be much less for most of the kits, and therefore, the phenol-chloroform extraction followed by alcohol precipitation is advisable. However, the purity of the DNA must be ensured by finding A260/A230 ratio which should be greater than 1.8. Lower A260/A230 values indicate contamination from solvents (e.g., phenol).

9. Purification with the lithium chloride precipitation solution provided in the mMESSAGE mMACHINE SP6 kit will also yield the same quantity of RNA. Refer to mMESSAGE mMACHINE manual for further standardization [http://kirschner.med.harvard.edu/files/protocols/Ambion_mMessageMachine.pdf].

10. Warm all cell culture media, PBS, and trypsin-EDTA in a water bath to 37 °C prior to adding to the cells.

11. The primers used for amplifying the nsP3 region of CHIKV in our study were:
   - CHIKV nsP3 N1 forward primer
     5’ AAGGCCGAATTCGGGCACCGTCGTACCGGGT AATAACGCA 3’
   - CHIKV nsP3 full reverse primer
     5’ GGTACCCTCGAGTCATTACCCACCTGCCCTGT CTAGTC 3’

   The PCR amplification with this primer set will yield an amplicon of 1.6 kb. The primers are designed to be highly specific and produce a single amplicon only. If other bands appear, further optimization can be carried out by gradient PCR or increasing the annealing temperature. Keep a nontemplate control for the PCR reaction.

12. The plaque assay for CHIKV uses Vero cells. For Sindbis virus, we use BHK-21 cells instead. The methodologies mentioned in steps 1 and 2 are the same. The plaques in BHK-21 are
more clearly visible, when staining the live cells using 1:10 dilution of 0.33% neutral red in DPBS. Add 1 mL of diluted neutral red on the top of the agar overlay and incubate at 37 °C in a 5% CO₂ incubator for 2 h and count the plaques using a light box. For neutral red staining, the plaques should be counted immediately or/within a period of 2–3 h.

13. The cells should be 90–95% confluent with few spaces at the time of infection. Usually 1 × 10⁵ cells were seeded overnight to obtain a monolayer the next day. The incubation time for culturing should not go beyond 24 h.

14. The 1.2% Bacto agar is autoclaved and stored at room temperature. The melted Bacto agar will solidify when cooled to room temperature. Therefore, take the overlay agar mixture from the 42 °C water bath just prior to use.

15. The mouse models differ for each strain of virus. To study the virulence of wild type versus mutants for CHIKV, we used 2-day old CD-1 pups. The same kind of virulence study for the TE strain of Sindbis virus can be done in 2-week old CD-1 mice.

16. Verify that the mice are adequately anesthetized by gently pinching the toes. If the animal withdraws their paw, anesthetize for few more minutes till a deep plane of sleep has been attained.

17. Place pups on a thermal mat to avoid a drop-in body temperature during anesthesia, and monitor until they recover.

18. Keep separate litters for morbidity/mortality studies and for a time course infection experiment involving the collection of organs and serum.

19. We use lysing matrix tubes with lysing matrix A (orange-capped tube having garnet matrix and ¼ ceramic sphere) for homogenizing the tissues for protein and tubes with lysing matrix D (green-capped tubes having 1.4 mm ceramic spheres) for homogenizing the tissues for RNA in QIAzol reagent using a MP FastPrep-24 homogenizer.

20. Make sure to take out the BD OptEIA TMB substrate kit to room temperature, at least 30 min prior to use. Combine equal amounts of reagent A and reagent B no more than 15 min prior to adding to the plates.

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