Chapter 21

Testing Antiviral Compounds in a Dengue Mouse Model

Wouter Schul, Andy Yip, and Pei-Yong Shi

Abstract

Dengue fever is an emerging mosquito-borne flaviviral disease that threatens 2.5 billion people worldwide. No clinically approved vaccine and antiviral therapy are currently available to prevent or treat dengue virus (DENV) infection. Vertebrate animals other than primates are not normally infectable with DENV; however, a small animal dengue infection model would greatly facilitate the development of a vaccine or an antiviral therapy. To this end, a rodent model for DENV infection has been established in IFN-α/β and IFN-γ receptor-deficient (AG129) mice. This chapter describes the protocol for the DENV infection model in AG129 mice and testing of antiviral compounds by oral gavage or parenteral injection.

Key words Flavivirus, Dengue virus, Animal model, Antiviral testing

1 Introduction

Dengue virus (DENV) belongs to the Flavivirus genus in the *Flaviviridae* family and consists of four serotypes. DENV is an enveloped virus, containing a single-stranded positive-sense RNA genome. The virus causes dengue fever (DF) which is a major worldwide health concern with significant economic cost. Two-fifths of the world's population lives in areas that are endemic to DENV, with an estimated 50 million infections per year. Approximately 500,000 cases progress to a more severe form of the disease called dengue hemorrhagic fever (DHF), often requiring hospitalization. Without proper treatment DHF can progress to dengue shock syndrome (DSS), which is often fatal [1–3].

Humans and *Aedes* mosquitoes are the main DENV host. Nonhuman primates (NHP) also serve as a reservoir of sylvatic DENV [4]. However, there is no laboratory animal model for dengue infection that faithfully reflects dengue disease at present. Rodents are not normally susceptible to DENV infection, but through the use of genetically modified mice several mouse infection models for dengue virus have been established. All these models rely on circumventing part of the murine innate immune system

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in some way, and the most reliable mouse infection models rely on a partial or a complete deficiency in the interferon response pathways.

A common genetically modified mouse strain used to establish DENV infection is the AG129 line which lacks both IFN-α/β and IFN-γ receptors in the 129/Sv background [5]. Johnson and Roehrig [6] were the first to report that DENV infection could be established in the AG129 mice and many research labs have subsequently used this system for further studies of dengue infection in vivo, including testing antiviral compounds. Other knockout mouse lines that have been shown to be infectable with DENV are A129 mice (deficient in the IFN-α/β receptors only) [7], and STAT1−/− mice [8]. It should be noted that even in the AG129 mice, which completely lack the ability to respond to type I and II interferons, not every strain of DENV can establish infection [9]. In our laboratory less than 10 % of the DENV serotype 2 (DENV2) strains could reproducibly establish an infection in the AG129 mice, and an even lower percentage was found for the three other serotypes (unpublished data). A DENV strain that can reliably infect AG129 mice can either be obtained through systematically testing all strains in a laboratory's collection (starting with the DENV2 strains) and/or by requesting a strain known to infect AG129 mice (e.g., strains TSV01, PL046, New Guinea C, WestPac74) from other laboratories.

Most strains known to infect the AG129 mice give rise to viremia, viral load in tissues, and inflammatory responses, but do not lead to overt signs of disease and animals will clear the infection within 4–7 days. However, a few strains have been generated through repeated adaptive selection that can cause severe symptoms and death after infection of AG129 mice: the D2S10 [10] and plaque-purified S221 clone [11], and the D220 strain [12]. Tan et al. [13] found that non-adapted DENV2 strain D2Y98P can also cause pathogenic and lethal infection in AG129 mice, and showed that this property is caused by a specific mutation in the viral NS4B protein which can be conferred to a nonpathogenic infectious strain by site-directed mutagenesis [14].

An additional phenomenon associated with DENV infection is known as antibody-dependent enhancement (ADE). In humans, immunity to one of the four DENV serotypes can increase disease severity upon subsequent infection with another serotype, caused by low-affinity cross-reactive antibodies. In 2010, Balsitis et al. [15] described ADE infection of AG129 mice with enhanced disease. This was achieved by injecting the AG129 mice with a low dose of cross-reactive polyclonal or monoclonal antibodies one day before DENV infection. Here we describe a well-established AG129 mouse model for testing the in vivo efficacy of antiviral compounds by oral gavage or parenteral injection.

2 Materials

2.1 Virus Production

1. C6/36 cells: ATCC (CRL-1660).
2. Virus: TSV01 (DENV2), NGC (DENV2; ATCC, VR-1255), Westpac74 (DENV1), D2S10 (mouse-adapted DENV2), D2Y98P (DENV2) (*see* **Note 1**).
3. Culture medium for C6/36 cells: RPMI 1640 medium (Gibco), 5 % fetal bovine serum (FBS), 1 % penicillin and streptomycin (P/S).

2.2 Mouse Infection

1. AG129 mice (*see* **Note 2**): Obtained from B&K Universal Ltd, Hull, UK, to establish a breeding colony. Male and female mice between 7 and 12 weeks of age.
2. Syringes: 0.5 mL insulin syringes with attached 29G needles (BD).

2.3 Dosing

Both stainless steel reusable or disposable straight needles can be used, depending on personal preference. Since mice between 7 and 12 weeks were used for infection, there is a wide range of body weights to be considered when choosing a suitable gavage needle. Younger mice usually weigh less than their older siblings.

1. Gavage needles for mice between 15 and 20 g body weights: 22G, 1 to 1.5 in. in length, 1.25 mm ball diameter.
2. Gavage needles for mice between 20 and 25 g body weights: 20G, 1–3 in. in length, 1.90–2.25 mm ball diameter.

2.4 Sampling

1. Anesthesia: Isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane) used in a calibrated isoflurane anesthesia machine suitable for small animals (e.g., VetEquip Mobile Laboratory Animal Anesthesia System).
2. Collection: Microhematocrit Capillary Tubes (Fisher, Cat. No. 22-362-574), Microtainer EDTA tubes (BD, Cat. No. 365974).

2.5 Sample Analysis

1. BHK-21 cells: ATCC (CRL-10).

2.5.1 Plaque Assay

2. Culture medium for BHK-21 cells: RPMI 1640 medium supplemented with 10 % FBS, 1 % P/S.
3. Plaque assay overlay: 0.8 % methyl cellulose with 2 % FBS:
   (a) Prepare 500 mL of 1.6 % methyl cellulose by dissolving methyl cellulose powder in water (slowly adding powder to water while stirring), autoclave, store overnight at 4 °C for hydration before use.
   (b) Prepare 500 mL of 2× RPMI 1640 by dissolving one bag of RPMI 1640 powder (for 1 L) in Millipore water. Add

20 mL FBS, 10 mL P/S, 5 mL $NaHCO_3$ (7.5 %), 25 mL of 1 M HEPES buffer. Filter through a 0.2 μm membrane.

(c) Mix 500 mL of 2× RPMI 1640 with 500 mL of 1.6 % methyl celllulose, then add 5 mL DMSO. Mix well, aliquot, and store at 4 °C until use.

4. 1 % (w/v) crystal violet solution: Dissolve 5 g crystal violet in 500 mL water, stir overnight until dissolved.

2.5.2 RT-PCR

1. SuperScript III Reverse Transcriptase (Invitrogen, Cat. No. 18080-044).
2. RNasin (40 U/μL) (Promega).
3. FastStart DNA Master^{PLUS} SYBR Green Kit (Roche Diagnostics, Cat. No. 03515869).
4. Glass capillaries (Roche Diagnostics).
5. QIAamp Viral RNA Mini Kit (Qiagen).

2.5.3 NS1 ELISA

Platelia Dengue NS1 Ag Kit (Bio-Rad, Cat. No. 72830).

2.5.4 Mouse Cytokines

1. Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD, Cat. No. 552364).
2. A dual-laser flow cytometer equipped with a 488- or 532-nm and a 633- or 635-nm laser capable of distinguishing 576-, 660-, and >680-nm fluorescence.

3 Methods

3.1 Preparing Virus Stocks (See Note 3)

1. Grow C6/36 cells in RPMI 1640 medium + 5 % FBS at 28 °C, to 70–90 % confluence in one or more tissue culture flasks. A cell count can be performed to get an estimate of the number of cells per flask.
2. Take one vial of DENV stock with known titer from −80 °C freezer and thaw rapidly. Dilute the virus in RPMI 1640 medium + 5 % FBS to result in a multiplicity of infection (MOI) of 0.01–0.1 plaque-forming units (PFU)/cell (i.e., a flask containing 10^7 cells receives 1 mL of 10^5–10^6 PFU of virus).
3. Incubate the infected cells at 28 °C for 3–7 days, depending on the growth characteristics of the DENV strain. Check the signs of increased cell death (cytopathic effect) daily as indication of viral proliferation although this may not be apparent for every DENV strain.
4. After 3–7-day incubation remove the culture medium from the infected cells and centrifuge at 5,000 × *g* for 5 min. Aliquot

the supernatant into cryovials, quickly freeze in liquid nitrogen, and store at −80 °C.

5. Thaw one vial to determine the viral titer by a plaque assay (*see* Subheading 3.5.1).

3.2 Infection of Mice by Parenteral Injection (See Note 4)

1. Transfer AG129 mice into cages according to their experimental group.
2. Take one or more vials of DENV stock from the −80 °C and thaw rapidly. Dilute the virus to an appropriate titer in RPMI1640 medium and let it warm to room temperature.

3.2.1 Intraperitoneal Injection of DENV

Intraperitoneal injection of 0.25 mL at 5×10^6 PFU/mouse or higher of a nonpathogenic DENV strain usually gives reproducible viremic infection. Therefore, the virus should be diluted to a titer of 2×10^7 PFU/mL.

The non-adapted pathogenic DENV strain D2Y98P can be injected intraperitoneally as low as 10^4 PFU/mouse to still give 100 % mortality although injection with higher titers gives a more robust and viremic infection [13].

3.2.2 Intravenous Injection of DENV

Mouse-adapted pathogenic DENV strains like D2S10 are typically injected intravenously in the tail vein. For the D2S10 strain the amount to be injected should be 10^7 PFU/mouse or higher (*see* **Note 5**).

3.3 Compound Dosing (See Note 6)

Compounds to be tested for their in vivo activities can be dosed either orally or by injection.

3.3.1 Injection of Compound

Completely dissolve the test compound in the vehicle fluid which is typically an aqueous buffer (e.g., PBS), or contains organic excipients allowed under animal testing protocols. The advantage of dosing by injection is that 100 % of the compound is delivered into the body allowing for maximal exposure; however, it will not reflect the oral bioavailability of a compound. Injection is typically by intravenous, subcutaneous, or intraperitoneal route.

3.3.2 Oral Dosing of Compound

1. Oral dosing of a compound is performed by oral gavage and takes gastrointestinal stability and permeability, extended period of absorption, and first-pass metabolism through the liver into account which is particularly relevant if ultimately an oral dengue drug is intended.
2. For oral dosing a compound can be either in solution or suspension.
3. Fill a 1 mL syringe with gavage needle with drug solution/suspension and carefully orally dose a specific volume (typically 0.1–0.2 mL) into the stomach of a mouse.

3.3.3 Dosing Schedule

The compound dosing schedule can vary from a single dose to multiple doses per day over several days, starting directly after DENV infection or one or several days after or before infection. Dosing levels are dependent on the expected exposure and in vitro potency of the compound but typically range from 3 to 300 mg/kg (i.e., milligram of compound per kilogram of mouse body weight) (*see* **Note 7**).

3.4 Sampling

1. Infected mice can be sampled daily for several days by retro-orbital puncture. Mice are anesthetized by isoflurane gas inhalation. A glass capillary is used to draw a small amount (0.1–0.2 mL) of blood from the retro-orbital plexus of the anesthetized mouse. The blood is collected into an EDTA tube. Alternatively, blood can be collected in tubes containing sodium citrate solution to give a final concentration of 0.4 % (*see* **Note 8**).
2. After sampling, the mouse is placed back into a cage. Make sure that it does not get hypothermic, e.g., by placing the cage on a warm pad or by wrapping the mouse with a tissue paper where it can recover from the effects of anesthesia.
3. Perform terminal bleeding similarly by retro-orbital puncture followed by cervical dislocation under isoflurane anesthesia.
4. Perform sample processing of anti-coagulated blood by immediate centrifugation in an Eppendorf centrifuge for 3 min at 7,000 × *g*. Remove the supernatant plasma carefully and transfer to a labeled cryo-tube. Subsequently, take 12 µL of plasma and add it to another labeled cryo-tube containing 588 µL of RPMI 1640 medium (effectively diluting 50×). Both tubes are then snap frozen in liquid nitrogen and stored at −80 °C for later analysis.

3.5 Sample Analysis

3.5.1 Plaque Assay

1. Using serum-free RPMI 1640 as diluent, perform a fivefold serial dilution on the 1:50 pre-diluted mouse plasma sample (*see* Subheading 3.4, **step 4**) in a sterile 96-well microtiter plate, resulting in 4 serially diluted samples at 1:50, 1:250, 1:1,250, and 1:6,250.
2. Carefully overlay 200 µL of each diluted sample onto BHK21 cells in a 24-well plate. Repeat for the remaining samples. Swirl the plate to ensure adequate mixing and incubate the plate at 37 °C, 5 % CO_2 for 1 h, briefly shaking the plate gently at the 30-min mark.
3. Remove virus inoculums with vacuum aspiration and overlay with 500 µL of 0.8 % methyl cellulose medium with 2 % FBS, taking care not to disturb the cell monolayer.
4. Incubate at 37 °C, 5 % CO_2 for 4 to 6 days depending on the strain of dengue virus used.

5. Gently rinse the plates several times in 3.7 % formaldehyde, and then soak for 1 h at room temperature. Shake plates (wells facing down) a few times to remove the methyl-cellulose layer.
6. Rinse plates in copious volumes of water in a large container. Shake plate (wells facing down) to remove excess water from the wells.
7. Add several drops of 1 % crystal violet into each well, enough to cover the cell monolayer, and set aside for 1 min.
8. Invert plate on layers of paper towels to remove excess crystal violet.
9. Rinse plates in copious volumes of water in a beaker. Shake plates (wells facing down) to remove excess water.
10. Dry plates in a 50 °C oven and count the number of plaques on the plates to determine the viral titer of each sample.

3.5.2 RT-PCR

1. Perform viral RNA isolation from the mouse plasma samples and appropriate standards (virus samples with known titer) with the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions.
2. Elute RNA in 60 μL of buffer AVE (supplied with the QIAGEN kit).
3. Synthesis of cDNA is performed using SuperScript III reverse transcriptase. The reaction mixture consists of the following:

RNase-free water	5 μL
Random hexamers (250 ng/μL)	1 μL
dNTPs (10 μM)	1 μL
RNA sample	6 μL

4. Heat the mixture to 65 °C for 5 min and place on ice immediately. Then add the following:

5× first-strand buffer	4 μL
DTT (100 mM)	1 μL
RNasin (40 U/μL)	1 μL
Superscript III enzyme	1 μL

5. Incubate the reaction mixture at 25 °C for 5 min and then at 42 °C for 60 min.
6. Stop the reaction by heating the mixture to 70 °C for 15 min.
7. Perform real-time PCR on a Roche LightCycler 2.0 system using the FastStart DNA MasterPLUS SYBR Green I kit.

Pipette the following reaction mixture into an Eppendorf tube for each sample:

SYBR Green I	4 µL
cDNA	2 µL
Primer 1 (10 µM) (*see* **Note 9**)	1 µL
Primer 2 (10 µM) (*see* **Note 9**)	1 µL
PCR-grade water	12 µL

8. Carefully pipette the reaction mixture into the glass capillaries.
9. Fit capillaries into precooled adapters and centrifuge at 700 × *g* for 5 s.
10. Transfer the capillaries from the rotor into the Lightcycler and begin the amplification using the following LightCycler PCR program:

    Denaturing and enzyme activation:

    10 min at 95 °C, slope 20 °C/s, acquisition mode: none

    Amplification:

    40 cycles of:

    5 s at 95 °C, slope 20 °C/s, acquisition mode: none

    5 s at 60 °C, slope 20 °C/s, acquisition mode: none

    20 s at 72 °C, slope 20 °C/s, acquisition mode: single

    Melting curve:

    1 s at 95 °C, slope 20 °C/s, acquisition mode: none

    5 s at 65 °C, slope 20 °C/s, acquisition mode: none

    0 s at 95 °C, slope 0.1 °C/s, acquisition mode: continuous

11. Use the cycle crossing point (Cp) values from the standards to generate a standard curve. The log-linear correlation between the Cp value and the virus concentration holds true over a range of 10^2 to 10^5 PFU/mL for all four dengue serotypes. With this information, the viral titer of the mouse samples can be determined.

3.5.3 NS1 ELISA

1. Carefully plan the plate layout for controls and samples.
2. Take the carrier tray and the strips out of the protective pouch, and leave at room temperature for a few minutes to allow temperature equilibration.
3. For the test samples, the dilution factor is 1:500, prepared by diluting 5 µL plasma in 45 µL PBS, mix well to get a 1:10

dilution, then add 1.2 μL from this 1:10 dilution mixture to 58.8 μL of R7 diluent, and mix well to get a 1:500 dilution.

4. For the control samples, use undiluted (neat), strictly following the indicated distribution sequence, and distribute successively in the wells:

   50 μL of diluent

   50 μL of samples (or controls)

   100 μL of diluted conjugate

5. Cover the reaction microplate with the supplied adhesive sealing film, pressing firmly onto the plate to ensure complete sealing.
6. Incubate the microplate in a 37°C incubator for 90 min.
7. Prepare the dilution of the washing solution.
8. At the end of the incubation period, carefully remove the adhesive sealing film. Remove the contents of all wells into a container for biohazard waste via vacuum aspiration. Wash the microplate 6 times with washing solution. Aspirate the washing solution. Leave the last round of washing solution in the wells.
9. Prepare the R8 + R9 solution as described in the user guide. Remove the remaining washing solution and place 160 μL of the R8 + R9 solution into each well. Allow reaction to develop at room temperature for 5 min.
10. Add 100 μL of the stop solution to each well by using the same sequence and rate of distribution as for the development solution.
11. Carefully wipe the plate bottom with lint-free tissue paper and read the optical density at 450/620 nm using an ELISA plate reader within 30 min after stopping the reaction.

3.5.4 Detection of Mouse Cytokines

A brief protocol is attached below; for more details check the user guide from the manufacturer.

1. Reconstitute mouse inflammation standards in assay diluent.
2. Dilute standards by serial dilutions using the assay diluent.
3. Mix 10 μL/test of each mouse inflammation capture bead suspension (vortex before aliquoting).
4. Transfer 50 μL of mixed beads to each assay tube.
5. Add standard dilutions and test samples to the appropriate sample tubes (50 μL/tube).
6. Add PE detection reagent (50 μL/tube) and incubate for 2 h at room temperature.
7. In the meantime, perform the cytometer setup bead procedure.

8. Add Cytometer setup beads (vortex before adding) to set up tubes A, B, and C (50 μL/tube).
9. Add 50 μL of FITC positive control to tube B and 50 μL of PE positive control to tube C.
10. Incubate for 30 min at room temperature, protected from light.
11. Add 400 μL of wash buffer to tubes B and C.
12. Add 450 μL of wash buffer to tube A.
13. Use tubes A, B, and C for cytometer setup as described in more detail in the supplied user guide.
14. After 2-h incubation, wash samples with 1 mL wash buffer and centrifuge at 200 × *g* for 5 min.
15. Add 300 μL of wash buffer to each assay tube and analyze samples.
16. Acquire the samples on a flow cytometer. For details, go to bdbiosciences.com/cbasetup and select the appropriate flow cytometer under CBA Kits: Instrument Setup.
17. Analyze BD CBA Mouse Inflammation Kit data using FCAP Array software. For instructions on analysis, go to bdbiosciences.com/cbasetup and see the Guide to Analyzing Data from BD CBA Kits, using FCAP Array software.

3.5.5 Histopathology

If needed, mouse liver and kidney can be harvested and fixed in 10 % formalin buffered in PBS, pH 7.4 for 2 days at room temperature before being sent for histopathology studies.

4 Notes

1. All procedures involving DENV should be performed according to the local biosafety regulations and protocols, including importation, storage, registration, and handling of the virus. In most countries, working with DENV needs to be conducted in a class II biosafety cabinet or higher. A risk assessment of the virus, the laboratory, and the procedures may be necessary. Researchers involved in biosafety and/or animal experiments should be evaluated for additional training needs, and should be made aware of all risks involved in the work.
2. All procedures involving mice require a protocol pre-approval by the Institutional Animal Care and Use Committee (IACUC). The investigator should have an experimental plan including the following:

(a) Calculation of the number of animals required per experimental group: This depends upon the level of variation observed between experimental animals, as well as the level of difference expected between control and experimental groups. In our laboratory we typically used 6 animals per group allowing statistically significant detection of five- to tenfold changes in viremia levels.

(b) A plan that clearly defined experimental end-points: All researchers working with infected mice should be able to recognize signs of severe illness, including hunched posture, ruffled coat, weight loss, and immobility. Mice are to be humanely terminated when they show signs of severe illness (method and signs to be agreed upon with the IACUC).

3. Production of high-titer stocks of DENV takes optimization and may vary for different strains. For some strains MOIs of 0.1–1 with 6–7 days of incubation may be required, while for other strains the optimal viral titer is reached after lower MOI infection or shorter incubation.

   DENV is grown in C6/36 mosquito cells at 28 °C which can be done by adjusting a 37 °C incubator to 28 °C. However, these incubators can be prone to overshooting the set temperature by one or more degrees, especially if the set temperature is close to room temperature and due to thermal insulation of the incubator stay at this elevated level for an hour or more resulting in poor cell and viral growth. It is therefore advisable to use a dedicated refrigerated incubator that can both heat and cool the incubation chamber to ensure incubation at the proper temperature.

4. Mouse handling and experimentation techniques: The experiments described in this chapter include references to mouse experimental techniques such as intraperitoneal and subcutaneous injections, tail vein injection, oral gavage, retro-orbital bleeding, and cervical dislocation. The details of these techniques are beyond the scope of these instructions and investigators are referred to mouse experimentation instruction manuals and hands-on training to learn more about these procedures.

5. Since intravenous injection of pathogenic virus requires small volumes (0.05–0.1 mL) containing large amounts of virus (10^7 PFU), this requires a stock with viral titer of 10^8 PFU/mL or higher, which can be challenging to achieve and require optimization of the viral production (*see* Subheading 3.1). Alternatively, concentrating the virus by (ultra)centrifugation can be considered although this often results in an overall loss of infectious virus up to 90 %. Therefore, a large starting volume of virus stock may be needed.

6. Pharmacokinetic compound profiling: It is advisable that, before starting dosing novel compounds to DENV-infected AG129 mice, a series of single-dose pharmacokinetic studies are conducted with novel compounds in uninfected wild-type 129/Sv mice to determine the plasma concentrations, half-life, rate of clearance, oral bioavailability, etc. In our experience, we did not observe any difference in pharmacokinetic properties of compounds between DENV-infected AG129 mice and uninfected wild-type 129/Sv mice (unpublished data). Such pharmacokinetic data is extremely valuable to optimize the dosing schedule (e.g., multiple dosing per day for a compound with a short half-life) or to interpret any (lack of) antiviral effect.
7. Dosing schedule options: Compound efficacy testing in DENV-infected AG129 mice can involve dosing several different compounds at a fixed dose for head-to-head comparison, or a dose–response experiment of a single compound at different dosing levels. In the latter case, a threefold increase over a relevant range is often applied (e.g., dosing at 3, 10, 30, 100 mg/kg or 5, 15, 50, 150 mg/kg). It is recommended to include a negative control group in each experiment that is dosed with the vehicle fluid, and a positive control group dosed with a known DENV inhibitor, e.g., *N*N-DNJ, BuCast [9], or NITD008 [17].
8. Do not use heparin or heparin-treated capillaries, tubes, or containers to anti-coagulate blood. Heparin can competitively prevent binding of DENV to heparan sulfates on the surface of cells blocking viral entry, interfering with viral infection assays, e.g., plaque assays.
9. PCR Primers

   For quantitative PCR detection of DENV genomes of all four serotypes, we have been using the D1 and D2 primers as described by Lanciotti et al. [16]. The primer sequences are as follows:

   D1 forward primer: 5′-TCAATATGCTGAAACGCGCGAG AAACCG-3′

   D2 reverse primer: 5′-TTGCACCAACAGTCAATGTCTTC AGGTTC-3′

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