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Optimized Protocol for the Amplification and Viral Titration of Dengue Virus Serotypes 1–4 (DENV1–4) / Protocolo optimizado para la amplificación y titulación de los virus del dengue serotipos 1–4 (DENV1–4) V.1

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We use this protocol and it's working

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Disclaimer

Este protocolo implica el manejo de virus infecciosos (Dengue virus), así como químicos potencialmente peligrosos (p. ej. formaldehído, cristal violeta). Solo debe ser realizado en laboratorios con instalaciones de bioseguridad adecuadas (BSL-2/BSL-3) y por personal entrenado, siguiendo las regulaciones institucionales y nacionales. Los autores y su institución no se hacen responsables por un uso inadecuado o no autorizado de este protocolo.

Abstract

This protocol describes the preparation, amplification, and verification of the four serotypes of Dengue virus (DENV 1-4) using BHK-21 and C6/36 cell lines. It includes cell culture conditions, viral amplification in C6/36 cells, titration by plaque reduction assay (plaque assay) in VERO CCL-81 cells, and confirmation of viral presence by RT-qPCR. In addition, it details viral stock storage conditions, biosafety recommendations, and the minimum traceability required for each batch. The protocol is designed to increase viral titers, improve their long-term stability, and ensure both the reproducibility of results and the compatibility of viral stocks for cellular infection studies as well as molecular and biochemical analyses.



Guidelines

All procedures involving the Dengue virus must be performed in a BSL-2 laboratory, within a Class II biosafety cabinet, and always using appropriate personal protective equipment (PPE), including a lab coat, gloves, mask, and eye protection. Only trained personnel should handle the virus. Institutional biosafety guidelines must be followed, and a detailed record of all viral stocks (batch, passage, date, titer, and responsible operator) should be maintained.

Materials

Equipment and Consumables

- Class II biosafety cabinet, type A2 (BSL-2) (BIOBASE, BSC-1300IIA2-X)
- CO₂ incubator, 37 °C, 5% CO₂ (Nuaire, 5820 Series 2)
- Non-CO₂ incubator, 28 °C (E&Q Lab)
- Refrigerated centrifuge for 15 mL tubes and Amicon filters (Orto Alresa, Digicen 21R)
- Refrigerated microcentrifuge for 1.5 mL tubes (Sigma 1-14K)
- Water bath, 37 °C (Branson 2800)
- Micropipettes (Sartorius)
- Filter tips, 1000 µL (BioSeen, SGA-22D)
- Filter tips, 200 µL (Thermo Fisher Scientific, 2069-HR)
- Filter tips, 10 µL (Thermo Fisher Scientific, 2139-HR)
- Serological pipettes, 5 mL (SPL, 91005)
- Serological pipettes, 10 mL (SPL, 91010)
- Sterile 1.5 mL tubes (Biologix, 80-1500)
- Sterile 15 mL tubes (SPL Life Sciences, 50015)
- Sterile 50 mL tubes (SPL Life Sciences, 50050)
- T25 culture flasks (NEST, 707003)
- T75 culture flasks (Corning, CLS430641U)
- 24-well plates (Nunc, 142475)
- Cryovials, 0.5 mL (USA Scientific, 1405-9710)
- Inverted optical microscope (Zeiss, Primovert)
- Real-time thermal cycler (Bio-Rad, CFX Opus 96)
- 0.1 µm filters (Santa Cruz, sc-358809)
- 0.2 µm filters (Santa Cruz, sc-358811)
- Isolate II RNA Mini Kit (Bioline)

Cells and Virus

- BHK-21 cell line (ATCC)
- C6/36 cell line (ATCC)
- Dengue virus 1-4 stocks

Media and Buffers

- RPMI 1640 1X (Gibco, 31800-022)
- Leibovitz's L-15 medium (Gibco, 41300-021)
- Heat-inactivated, low-endotoxin fetal bovine serum (FBS) (Capricorn Scientific, FBS-GI-12A)
- L-glutamine, 200 mM (Life Technologies, G7513-100 mL)
- Penicillin (100 U/mL) / Streptomycin (100 µg/mL) (Capricorn Scientific, PS-B)
- Tryptose phosphate (Life Technologies, 8782)
- PBS 1X (without Ca²⁺/Mg²⁺) (ChemCruz, sc-24946)
- HBS (20 mM HEPES, 150 mM NaCl, pH 7.4)
- Nuclease-free ultrapure water (Sigma, W4502)



- Trypsin-EDTA 0.25% (Capricorn Scientific, TRY-3B)
- HEPES (Sigma, H0887-100 mL)
- Carboxymethylcellulose, medium viscosity (Sigma, C4888-500G)
- Sodium bicarbonate, 7.5% (Gibco, 25080-094)
- Formaldehyde 4% in PBS 1X (PanReac AppliChem, 131328-1211)
- Crystal violet (Albor Químicos, 12214)
- Bovine serum albumin (BSA) (GoldBio, A420-50)
- Luna Universal One-Step RT-qPCR Kit, SYBR Green (New England Biolabs, E3005S)
- SuperScript III Reverse Transcriptase (Thermo Fisher, two-step strategy only, Ref: 18080085)

Primers (final concentration 0.2 μ M in qPCR)

- DENV_7764_Fwd: 5'-CGTCGAGAGAAATATGGTCACACC-3'
- DENV_7844_Rev: 5'-CCACAATAGTATGACCAGCCT-3'
- hGAPDH_Fwd (optional): 5'-TGTTGCCATCAATGACCCCTT-3'
- hGAPDH_Rev (optional): 5'-CTCCACGACGTACTCAGCG-3'

Safety warnings

1. Dengue virus is a Risk Group 2 pathogen; handle with caution to prevent laboratory-acquired infections.
2. Avoid the generation of aerosols when pipetting or discarding viral material.
3. Formaldehyde (4%) is toxic and irritating; crystal violet is a potentially mutagenic agent. Handle both using gloves, a laboratory coat, and eye protection.
4. Dispose of all biological waste according to institutional biosafety regulations (autoclaving or chemical inactivation).
5. Do not reuse thawed viral aliquots; they must be discarded after a single use.

Ethics statement

This protocol involves exclusively the use of the Dengue virus (DENV 1-4) in established cell lines (BHK-21 and C6/36), with no use of animals or human samples. All activities must be performed under Biosafety Level 2 (BSL-2) regulations, in full compliance with national and institutional biosafety and occupational health standards. Personnel carrying out this procedure must be trained in handling viral agents, managing hazardous biological waste, and using appropriate personal protective equipment. Adherence to these standards ensures both operator safety and the ethical validity of the experimental work.



Before start

- Perform all procedures in a BSL-2 laboratory using a Class II biosafety cabinet and proper personal protective equipment (lab coat, gloves, eye protection, mask).
- Use small virus aliquots to avoid repeated freeze–thaw cycles; store stocks in cryovials at $-80\text{ }^{\circ}\text{C}$ or in liquid nitrogen (LN_2).
- Maintain rigorous records of viral batches, passages, titers, and storage conditions.
- Verify the availability of all required materials, reagents, and consumables (see materials list).
- Prepare complete media, buffers, and overlay solutions in advance, ensuring sterility and freedom from endotoxins and nucleases.
- Confirm that incubators are calibrated ($37\text{ }^{\circ}\text{C}$ with $5\% \text{ CO}_2$; $28\text{ }^{\circ}\text{C}$ without CO_2).
- Clean and disinfect biosafety cabinets before use.
- Label cryovials and plan storage locations prior to viral amplification.
- Confirm the availability of appropriate negative and positive controls for plaque assays and RT-qPCR (e.g., uninfected cultures, NTCs).
- Verify calibration of the qPCR thermocycler and micropipettes.
- Ensure nuclease-free conditions are maintained when handling RNA.

Introduction

- 1 Dengue virus (DENV) is an arbovirus belonging to the *Flaviviridae* family and transmitted primarily by *Aedes aegypti* and *Aedes albopictus* mosquitoes. It is a major global health problem, with approximately 40% of the world's population at risk and an estimated 50 million infections annually (1,2). In Colombia and other endemic regions, the detection of *Aedes* mosquitoes at unusually high altitudes has raised concerns about the impact of climate change and the potential expansion of transmission into new geographic areas (3). DENV exists as four well-established serotypes (DENV-1 to DENV-4), with a fifth serotype (DENV-5) recently reported (4). Infection can range from asymptomatic or mild febrile illness to severe clinical forms such as plasma leakage, hemorrhage, and multi-organ involvement, representing a significant cause of morbidity and mortality worldwide (5).
In research, viral amplification and titration protocols for DENV frequently result in high variability in infectious titers, influenced by factors such as cell passage history, media formulations, and methods of concentration. Ultracentrifugation, although widely used, is costly and not feasible for many laboratories, contributing to inconsistent yields of infectious particles (6). Therefore, the development of standardized, accessible, and reproducible protocols for DENV amplification and titration across serotypes 1–4 is critical to ensure experimental reproducibility and support downstream applications in diagnostics, pathogenesis studies, and antiviral research.

Materials and Reagents

- 2 **Equipment and Consumables**
 - Class II biosafety cabinet, type A2 (BSL-2) (BIOBASE, BSC-1300IIA2-X)
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 - Non-CO₂ incubator, 28 °C (E&Q Lab)
 - Refrigerated centrifuge for 15 mL tubes and Amicon filters (Orto Alresa, Digicen 21R)
 - Refrigerated microcentrifuge for 1.5 mL tubes (Sigma 1-14K)
 - Water bath, 37 °C (Branson 2800)
 - Micropipettes (Sartorius)
 - Filter tips, 1000 µL (BioSeen, SGA-22D)
 - Filter tips, 200 µL (Thermo Fisher Scientific, 2069-HR)
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 - Sterile 50 mL tubes (SPL Life Sciences, 50050)

- T25 culture flasks (NEST, 707003)
- T75 culture flasks (Corning, CLS430641U)
- 24-well plates (Nunc, 142475)
- Cryovials, 0.5 mL (USA Scientific, 1405-9710)
- Inverted optical microscope (Zeiss, Primovert)
- Real-time thermal cycler (Bio-Rad, CFX Opus 96)
- 0.1 µm filters (Santa Cruz, sc-358809)
- 0.2 µm filters (Santa Cruz, sc-358811)
- Isolate II RNA Mini Kit (Bioline)

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- C6/36 cell line (ATCC)
- Dengue virus 1-4 stocks

Media and Buffers

- RPMI 1640 1X (Gibco, 31800-022)
- Leibovitz's L-15 medium (Gibco, 41300-021)
- Heat-inactivated, low-endotoxin fetal bovine serum (FBS) (Capricorn Scientific, FBS-GI-12A)
- L-glutamine, 200 mM (Life Technologies, G7513-100 mL)
- Penicillin (100 U/mL) / Streptomycin (100 µg/mL) (Capricorn Scientific, PS-B)
- Tryptose phosphate (Life Technologies, 8782)
- PBS 1X (without Ca²⁺/Mg²⁺) (ChemCruz, sc-24946)
- HBS (20 mM HEPES, 150 mM NaCl, pH 7.4)
- Nuclease-free ultrapure water (Sigma, W4502)
- Trypsin-EDTA 0.25% (Capricorn Scientific, TRY-3B)
- HEPES (Sigma, H0887-100 mL)
- Carboxymethylcellulose, medium viscosity (Sigma, C4888-500G)
- Sodium bicarbonate, 7.5% (Gibco, 25080-094)
- Formaldehyde 4% in PBS 1X (PanReac AppliChem, 131328-1211)
- Crystal violet (Albor Químicos, 12214)
- Bovine serum albumin (BSA) (GoldBio, A420-50)
- Luna Universal One-Step RT-qPCR Kit, SYBR Green (New England Biolabs, E3005S)
- SuperScript III Reverse Transcriptase (Thermo Fisher, two-step strategy only, Ref: 18080085)

Primers (final concentration 0.2 µM in qPCR)

- DENV_7764_Fwd: 5'-CGTCGAGAGAAATATGGTCACACC-3'
- DENV_7844_Rev: 5'-CCACAATAGTATGACCAGCCT-3'
- DENV NS5 primers (DENV_7764_Fwd and DENV_7844_Rev) were previously reported in the literature by Xie et al., 2013. It is recommended to use a constitutive



housekeeping gene as a reference to normalize relative RNA expression in assays where such normalization is required.

Protocol

3 BHK-21 Cell Culture

The baby hamster kidney epithelial cell line (BHK-21) will be used to determine the viral titers of dengue virus (DENV) serotypes 1 to 4.

1. Thaw cryopreserved cell vials stored in liquid nitrogen (- 196 °C). Perform all steps in a Class II biosafety cabinet (BSL-2).
2. Place the vial in a 37 °C water bath until completely thawed.
3. Centrifuge the contents at 150 × g for 5 min.
4. Resuspend the pellet in 10 mL of growth medium (RPMI 1640 1X) supplemented with:
10% heat-inactivated
Low-endotoxin fetal bovine serum (FBS)
2 mM L-glutamine
Penicillin/Streptomycin 1X.

5. Seed the cells into T-75 flasks and incubate at 37 °C in a humidified atmosphere (60%) with 5% CO₂. If the initial cell number is low, seed into T-25 flasks instead.

Alternative option:

Dilute thawed cells directly, without centrifugation, in 10 mL of complete RPMI 1640 1X medium as described above. Incubate at 37 °C in a humidified atmosphere (60%) with 5% CO₂ and replace the medium after 2–3 h to remove dimethyl sulfoxide (DMSO).

6. Cells should be subcultured once they reach 80–90% confluence. The procedure is as follows:

Remove the flasks from the incubator and, inside the biosafety cabinet, wash the cells three times with PBS 1X (pH 7.4).

Add 0.25% trypsin-EDTA and incubate at 37 °C to detach the cells.

Reseed the cells into new flasks at a split ratio of 1:5 to 1:10, depending on cell density.

Note:

All reagents must be equilibrated to room temperature before contact with the cells.
Only use cells with a passage number <30.

4 C6/36 Cell Culture

The C6/36 cell line will be used for Dengue virus (DENV 1-4) amplification.

1. Thaw cryopreserved cell vials stored in liquid nitrogen (- 196 °C) inside a Class II biosafety cabinet (BSL-2).

2. Place the vial in a 37 °C water bath until completely thawed.
3. Centrifuge the vial at 150 × g for 5 min.
4. Resuspend the pellet in 10 mL of Leibovitz's L-15 medium supplemented with:
 - 10% fetal bovine serum (FBS)
 - 2 mM L-glutamine
 - Penicillin/Streptomycin 1X
 - 10% tryptose phosphate
5. Seed the cells into T-75 flasks and incubate at 28 °C in the absence of CO₂. If the initial number of cells is low, seed into T-25 flasks instead.

Alternative option: Dilute thawed cells directly, without centrifugation, in 10 mL of complete Leibovitz's L-15 medium. Incubate at 28 °C in the absence of CO₂ and replace the medium after 2–3 h to remove dimethyl sulfoxide (DMSO).

6. Cells should be subcultured once they reach 80–90% confluence to expand the culture. The procedure is as follows:
 - Remove the flasks from the incubator and, inside the biosafety cabinet, gently tap the flask three times to detach the cells.
 - Transfer the cells to new flasks using gentle pipetting (trypsinization is not required).
 - Reseed the cells into new flasks at a split ratio of 1:3 to 1:4, depending on cell density.

Note:

- All reagents must be equilibrated to room temperature before contact with the cells.
- Only use cells with a passage number <20.

5 Virus

- The viruses used were previously stored at –80 °C and titrated at the Laboratory of Virology and Molecular Biology, Corporación Universitaria Empresarial Alexander von Humboldt.
- The identity and quality of the viral strains were verified by RT-qPCR prior to experimental use.
- Previous infections to generate these working stocks were carried out using 500 µL of previously established viral batches, followed by monitoring until cytopathic effect (CPE) was observed.

6 Amplification of DENV 1–4 in C6/36 Cells

Preparation of cells for infection

1. Seed 1×10^7 C6/36 cells into T-25 culture flasks, 24 h prior to infection.
2. Maintain cells in Leibovitz L-15 medium (1×) supplemented with:
 - 2% FBS.
 - 2 mM L-glutamine (1%).
 - Pen/Strep 1× (penicillin 100 U/mL and streptomycin 100 µg/mL).
3. Incubate at 28 °C in a CO₂-free incubator until infection.

6.1 Preparation of the viral inoculum

1. In a biosafety cabinet, prepare 3 mL of viral inoculum in Leibovitz L-15 medium (1×) supplemented with 10% tryptose phosphate.
2. Use the viral stock previously stored in the laboratory, adjusting the number of viral particles according to the available titer.
3. Infect cells at a multiplicity of infection (MOI) of:
 - 0.1 ($\approx 1 \times 10^6$ viral particles for 1×10^7 cells) for DENV-1 and DENV-2.
 - 0.01 ($\approx 1 \times 10^5$ viral particles for 1×10^7 cells) for DENV-3 and DENV-4.

6.2 Cell Infection

1. Remove the culture medium from T-25 flasks with C6/36 cells.
2. Wash the monolayer three times with PBS (1×) to remove serum and antibiotics.
3. Add the prepared viral inoculum onto the cell monolayer.
4. Incubate at 28 °C for 2 h, gently swirling every 30 min to enhance viral adsorption.
5. After adsorption, remove and discard the inoculum following biosafety procedures.
6. Add maintenance Leibovitz L-15 medium (1×) without antibiotics.
7. Incubate at 28 °C in the absence of CO₂ for 6–7 days, or until partial cytopathic effect (CPE) is observed (e.g., syncytium formation) without complete monolayer destruction.

Note: T-75 flasks are not recommended for amplification; T-25 flasks provide significantly better viral titers.

6.3 Virus harvest

1. Once characteristic CPE is observed without monolayer destruction, collect the supernatant (without lysing the cells) into a sterile 15 mL tube.
2. Centrifuge at 4000 × g for 10 min to remove cellular debris.
3. Filter the supernatant through 0.1 µm membranes.
4. Aliquot into 200 µL volumes in sterile 0.5 mL cryovials.
5. Store at –80 °C. Each vial must be clearly labeled with at least the following information:
 - Virus/serotype – strain (if applicable) — host cell used (e.g., C6/36).
 - Batch (consistent format), viral passage number.
 - Date (YYYY-MM-DD), aliquot #/total (e.g., 03/24), operator initials.Maximum of three lines.

Example label:

DENV-2 (NGC) – C6/36
Batch 3.1-25 P=2
2025-09-10 A03/30 DPR

Notes:

- Always use cells with passage number <20 to avoid loss of viral susceptibility.

- Confirm viral identity and concentration using RT-qPCR and plaque assay in BHK-21 cells before subsequent experiments.
- Avoid freezing before aliquoting; pre-freeze steps may reduce viral titers, increase contaminants, and generate higher proportions of non-infectious particles.
- Cryovials provide better long-term viral stability compared to tubes.
- If needed, viral concentration can be performed using Amicon Ultra 100 kDa filters, or the buffer can be exchanged to HBS (10 mM HEPES, 150 mM NaCl) supplemented with stabilizers such as FBS, bovine serum albumin, or sugars like trehalose or sucrose, depending on downstream applications.

7 **Determination of Viral Titers by Plaque Reduction Assay (Plaque Assay) in BHK-21 Cells**

Cell seeding (24 h prior to the assay)

- Seed 1×10^5 BHK-21 cells/well in 24-well plates to reach ~90–100% confluence at the time of infection.
- Maintain in RPMI-1640 medium (1×) and incubate at 37 °C, 5% CO₂.

Note: A uniform monolayer is critical for obtaining well-defined plaques. If confluence is not optimal, adjust the seeding density in subsequent runs.

7.1 **Preparation of viral dilutions and cells**

Prepare 6 serial 10-fold dilutions (10^{-1} to 10^{-6}) using 1.5 mL tubes with unsupplemented RPMI-1640 medium:

- 900 µL medium + 100 µL virus = 10^{-1} dilution.
- Transfer 100 µL from the 10^{-1} tube into a new tube containing 900 µL medium = 10^{-2} dilution.
- Repeat sequentially up to 10^{-6} .
- Keep dilutions on ice or at 4 °C until use.
- Wash cells 2–3 times with PBS (1×) to remove serum residues that interfere with viral adsorption.
- Add each dilution to the corresponding wells (plate should be pre-labeled to avoid errors).
- Incubate at 37 °C, 5% CO₂ for 1–2 h to allow viral adsorption.
- Work in triplicate for each dilution.

Note:

- Avoid drying of the monolayer
- keep adsorption times uniform
- Gently agitate every 15–30 min to facilitate viral contact with the cells.

7.2 **Alternative protocol (optional)**

Perform the dilutions directly on the cells in the 24-well plate.

- After washing the cells, add 180 μL of unsupplemented medium per well across the entire plate.
- In the first well, add 20 μL of virus to obtain the first dilution (10^{-1}). Mix gently using circular and back-and-forth movements.
- Transfer 20 μL from this dilution to the next well containing 180 μL of medium to obtain the 10^{-2} dilution, and continue successively up to 10^{-6} .
- Incubate at 37 °C, 5% CO_2 for 1–2 h to allow viral adsorption.
- Perform each dilution in triplicate.
- After adsorption, remove the inoculum and discard according to biosafety guidelines.

7.3 **Add overlay medium**

Add 1 mL/well of overlay prepared as follows (for 50 mL total):

- RPMI-1640 2X.
- Carboxymethylcellulose (CMC, medium viscosity) 1.5% final (prepared as a 3% stock in distilled water).
- FBS 2% (1 mL).
- Sodium bicarbonate 2–3% (adjust according to pH/ CO_2 balance of incubator). If the medium already contains bicarbonate, add less.
- Pen/Strep 1 $\times$ (optional).
- Incubate at 37 °C, 5% CO_2 for 7 days (or 5–6 days for DENV-4).

Note: Dilutions correspond to a decimal series (1:10).

7.4 **Fixation and staining:**

- Carefully remove the overlay by inverting the plate and add 4% formaldehyde in 1X PBS to each well. Incubate for 30–60 min at room temperature.
- Remove the fixative and wash thoroughly with tap water to eliminate viscous residues (ensure that no residues remain, as this will affect staining and subsequent plaque counting).
- Add crystal violet solution and incubate for 30–60 min at room temperature.
- Wash with tap water to remove excess dye and allow the plate to air-dry.

Plaque counting and calculation of viral titers (PFU/mL):

- Select wells containing 10–20 plaques to ensure accuracy. Always confirm that the plaque counts are consistent with adjacent dilutions (e.g., if dilution 10^{-5} yields 20 plaques, dilution 10^{-6} should yield ~2 plaques, and dilution 10^{-4} should yield ~200 plaques).
- Count plaques per well and calculate the mean from duplicates or triplicates.
- Determine the viral titer using the following formula:

$$\text{PFU/mL} = \frac{\# \text{placas} \times \text{factor de dilución}}{\text{volumen de inóculo (mL)}}$$

Example: 45 plaques at 10^{-4} with 0.200 mL inoculum

$$\text{PFU/mL} = \frac{45 \times 10^4}{0,200} = 2,25 \times 10^6 \text{ PFU/mL}$$

Notes: Use the mean plaque count from replicates; report with 2–3 significant figures and include the dilution and inoculum volume used.

8 Verification of DENV by RT-qPCR

Samples and RNA

- Use viral supernatant/stock (from C6/36 cultures) for extraction.
- Extract viral RNA (e.g., Isolate II RNA Mini Kit) and elute in 30–50 μL nuclease-free water.
- Keep on ice; avoid repeated freeze–thaw cycles.

Primers (final concentration 0.2 μM)

Target gene: NS5 (DENV)

- DENV_7764_Fwd: 5'-CGTCGAGAGAAATATGGTCACACC-3'
- DENV_7844_Rev: 5'-CCACAATAGTATGACCAGCCT-3'

(Housekeeping controls such as hGAPDH are not used when working with viral supernatants.)

8.1 Reaction setup (final volume 20 μL per well)

- 10.0 μL Luna Universal One-Step Reaction Mix (2 $\times$)
- 1.0 μL Luna WarmStart® RT Enzyme Mix (20 $\times$)
- 0.4 μL forward primer (10 μM $\rightarrow$ 0.2 μM final)
- 0.4 μL reverse primer (10 μM $\rightarrow$ 0.2 μM final)
- 1–5 μL RNA (typically 1–2 μL). If RNA concentration is known: use 1–50 ng total RNA per 20 μL reaction. If RNA is not quantified: add the maximum permissible volume without compromising the mix, usually 1–5 μL eluate per reaction (keeping the final volume at 20 μL). Start with 2 μL ; if Ct is high (>35) or no signal is observed, increase up to 5 μL .

- Add nuclease-free water to 20.0 μL total.

Thermal cycling profile

- Reverse transcription: 55 $^{\circ}\text{C}$, 10 min
- Initial denaturation: 95 $^{\circ}\text{C}$, 1 min

40 cycles:

- 95 $^{\circ}\text{C}$, 10–15 s
- 60 $^{\circ}\text{C}$, 30 s
- Melting curve: 65–95 $^{\circ}\text{C}$, 0.5 $^{\circ}\text{C}$ increments (confirm single amplicon).

Plate layout and replicates

- Run technical duplicates or triplicates per sample.
- Include a standard curve (serial dilutions of RNA/cDNA reference) if quantification is desired.

Controls per run

- **NTC (No-Template Control):** nuclease-free water instead of RNA.
- **Mock:** RNA from uninfected culture supernatant (biological negative control).
- **Extraction blank:** tube processed through the extraction without a sample (detects kit/process contamination).

Note: –RT control is not applicable in one-step. If working with reference genes and DNA contamination is a concern, run parallel reactions omitting RT Enzyme Mix.

8.2 Interpretation and acceptance criteria

- Specific amplification: sigmoidal curve and single peak in melting analysis.
- Reproducibility: $\Delta\text{Ct} \leq 0.5$ between replicates.
- Negatives (NTC/Mock/blank): no specific amplification (or very late Ct with no defined peak).
- Report Ct (NS5) per sample; if a standard curve is included, report as copies/ μL or GE/mL.

Notes

- Maintain primer concentration at 0.2 μM ; if primer dimers appear, adjust to 0.15–0.2 μM or increase annealing temperature to 62–64 $^{\circ}\text{C}$.
- Always use filter tips and separate areas for pre-mix and RNA handling.
- Store RNA at –80 $^{\circ}\text{C}$; avoid more than one freeze–thaw cycle.
- For highly diluted samples, increase RNA input (up to 5 μL), adjusting water to keep the final volume at 20 μL .

(Optional) Master mix preparation for N reactions

- Calculate per-reaction volumes and scale to (N + 10%) to account for pipetting losses.
- Mix Reaction Mix (2 $\times$), RT Enzyme Mix (20 $\times$), and primers in bulk.

- Aliquot into wells and add RNA last, on ice.

Protocol references

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