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Optimized protocol for the amplification and viral titration of Zika Virus / Protocolo optimizado para la amplificación y titulación del virus del Zika

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We use this protocol and it's working

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Disclaimer

This protocol involves the handling of infectious viruses such as Zika virus, as well as potentially hazardous chemicals (e.g., formaldehyde, crystal violet). It must only be performed in laboratories with appropriate biosafety facilities (BSL-2/BSL-3) and by trained personnel, in compliance with institutional and national regulations. The authors and their institution are not responsible for any misuse or unauthorized application of this protocol.

Abstract

This protocol describes the preparation, amplification, and verification of Zika virus (ZIKV) using VERO CCL-81 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 Zika 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

- Vero CCL-81 cell line (ATCC)
- C6/36 cell line (ATCC)
- Zika virus stocks

Media and Buffers

- DMEM 1X (Gibco, 12800-082)
- 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) as mentioned by Giraldo *et al.* (6)

- Forward: 5'-CCGCTGCCCAACACAAG-3'
- Reverse: 5'-CCACTAACGTTCTTTTGCAGACAT-3'
- hGAPDH_Fwd (optional): 5'-TGTTGCCATCAATGACCCCTT-3'
- hGAPDH_Rev (optional): 5'-CTCCACGACGTACTCAGCG-3'

Safety warnings



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

Ethics statement

This protocol involves exclusively the use of the Zika virus in established cell lines (VERO CCL-81 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 Zika virus (ZIKV) is an arbovirus transmitted by *Aedes* mosquitoes, with a single-stranded RNA genome (1,2). It was first described in 1947 in Uganda and gained global attention in 2015 due to outbreaks in Brazil, which were associated with microcephaly and Guillain–Barré syndrome (3). In 2016, the World Health Organization (WHO) declared ZIKV a public health emergency of international concern (4). Unlike other flaviviruses, ZIKV has been linked to congenital infections, Guillain–Barré syndrome, and testicular atrophy. Clinical manifestations include fever, rash, and headache (5,6), and the expansion of the *Aedes* vector is influenced by climate change, contributing to the spread of ZIKV in new geographic regions (7). In ZIKV research, amplification protocols often yield variability in infectious viral titers due to multiple factors, including cell line passage history, culture conditions, and differences in media formulations. Similarly, viral concentration methods vary, with some approaches—such as ultracentrifugation—being costly and unavailable in many laboratories, which complicates the reproducibility of experiments and may hinder the interpretation of results (8,9). Based on these considerations, the **objective** of this work is to optimize a standardized and reproducible protocol for ZIKV amplification.

Materials and Reagents

- 2 **Equipment and Consumables**
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 - CO₂ incubator, 37 °C, 5% CO₂ (Nuaire, 5820 Series 2)
 - Non-CO₂ incubator, 28 °C (E&Q Lab)
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 - 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)
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 - Serological pipettes, 10 mL (SPL, 91010)
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 - 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)
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- Isolate II RNA Mini Kit (Bioline)

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- Zika virus stocks

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- 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)

- Forward: 5'-CCGCTGCCCAACACAAG-3'
- Reverse: 5'-CCACTAACGTTCTTTTGCAGACAT-3'
- hGAPDH_Fwd (optional): 5'-TGTTGCCATCAATGACCCCTT-3'
- hGAPDH_Rev (optional): 5'-CTCCACGACGTACTCAGCG-3'

Protocol

3 VERO CCL-81 Cell Culture

The VERO CCL-81 cell line, derived from the kidney cells of the African green monkey (*Chlorocebus sabaeus*), will be used to determine Zika virus (ZIKV) titers.

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 (DMEM 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 DMEM 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 Zika virus (ZIKV) 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 ZIKV Amplification in C6/36 Cells

Preparation of cells for infection

1. Seed 1×10^7 C6/36 cells in T-25 culture flasks, 24 h before infection.
2. Maintain the cells in maintenance medium (Leibovitz's L-15 1X) supplemented with:
 - 2% fetal bovine serum (FBS)
 - 2 mM L-glutamine (1%)
 - Penicillin/Streptomycin 1X
3. Incubate at 28 °C in a non-CO₂ incubator until infection.

6.1 Preparation of viral inoculum

1. In a biosafety cabinet, prepare 3 mL of viral inoculum in Leibovitz's L-15 1X medium supplemented with 10% tryptose phosphate.
2. Use previously stored ZIKV stocks, adjusting the number of viral particles according to the available titer.
3. Infect the cells at a multiplicity of infection (MOI) of 0.1 ($\approx 1 \times 10^6$ viral particles per 1×10^7 cells).

6.2 Cell infection

1. Remove the culture medium from the T-25 flasks containing C6/36 cells.
2. Wash the monolayer gently three times with PBS 1X to remove residual serum and antibiotics.
3. Add the prepared viral inoculum to the cell monolayer.
4. Incubate at 28 °C for 2 h, gently swirling the flask every 30 min to facilitate viral adsorption.
5. After adsorption, remove the inoculum and discard it following biosafety guidelines.
6. Add maintenance medium (Leibovitz's L-15 1X) without antibiotics.
7. Incubate at 28 °C in the absence of CO₂ for 6–7 days, or until partial cytopathic effect (CPE) is observed without complete monolayer destruction.

Note: T-75 flasks are not recommended for amplification. Using T-25 flasks consistently improves viral titers.

6.3 Virus harvest

1. Remove the flasks from the incubator and collect the supernatant into sterile 15 mL tubes, avoiding cell lysis.
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 (maximum three lines):
 - Virus/strain (if applicable) — cell line used for amplification (e.g., C6/36)
 - Batch number (consistent format), virus passage number
 - Date (YYYY-MM-DD), aliquot #/total (e.g., A03/24), operator initials

Example label:

ZIKV (MR766) – C6/36
Batch 4.2-25 P=3
2025-09-10 A03/30 ARS

Notes:

- Always use cells with a passage number <20 to avoid loss of viral susceptibility.
- Confirm the identity and concentration of amplified virus by RT-qPCR and plaque assay in VERO cells prior to experimental use.
- Avoid freezing the culture before aliquoting, as this may decrease viral titers, increase contaminants, and generate more non-infectious particles.
- Using cryovials improves viral titer stability over time.
- If necessary, concentrate the virus with Amicon-Ultra 100 kDa filters or transfer to a buffer such as HBS (10 mM HEPES, 150 mM NaCl) supplemented with a stabilizer (e.g., FBS, bovine serum albumin, trehalose, or sucrose), depending on the downstream applications.

7 Viral Titer Determination by Plaque Assay in VERO CCL-81 Cells

Cell seeding (24 h before the assay):

- Seed 1×10^5 VERO cells per well in 24-well plates to achieve ~90–100% confluence at the time of the assay.
- Maintain in DMEM 1X (maintenance medium) and incubate at 37 °C, 5% CO₂.

Note: A uniform monolayer is essential to obtain well-defined plaques. If the desired confluence is not reached, adjust the seeding density in the next run.

7.1 Preparation of viral dilutions and cells:

Prepare six 10-fold serial dilutions (10^{-1} to 10^{-6}) in 1.5 mL tubes using unsupplemented DMEM 1X as follows:

- 900 µL medium + 100 µL virus = 10^{-1} dilution.
- Transfer 100 µL of the 10^{-1} dilution into a new tube containing 900 µL medium = 10^{-2} dilution.
- Repeat successively until reaching 10^{-6} .
- Keep prepared dilutions on ice or at 4 °C until use.
- Wash the cells 2–3 times with PBS 1X to remove serum residues that may interfere with viral adsorption.
- Add each dilution to the designated wells (pre-marked to avoid misinterpretation).
- Incubate at 37 °C, 5% CO₂ for 1–2 h to allow viral adsorption.
- Perform each dilution in triplicate.

Note:

- Prevent the monolayer from drying.
- maintain uniform adsorption times.
- 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% CO₂ 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):

- DMEM 2X
- CMC (carboxymethylcellulose, medium viscosity) to a final concentration of 1.5% (prepared as a 3% stock solution in distilled water)
- 2% FBS
- Add bicarbonate to 1X if the medium does not already contain it
- Pen/Strep 1X (optional)

Incubate at 37 °C, 5% CO₂ for 5 days.

Note: The 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 ZIKV by RT-qPCR

Samples and RNA

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

Primers (final concentration 0.2 µM)

- Forward: 5'-CCGCTGCCCCAACACAAG-3'
- Reverse: 5'-CCACTAACGTTCTTTTGCAGACAT-3'

Note: (hGAPDH is not used when working with viral supernatants).

8.1 Reaction setup (final volume 20 µL/well)

- 10.0 µL Luna Universal One-Step Reaction Mix (2×)
- 1.0 µL Luna WarmStart® RT Enzyme Mix (20×)
- 0.4 µL Forward primer 10 µM → 0.2 µM final
- 0.4 µL Reverse primer 10 µM → 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 profile

- RT: 55 °C, 10 min
- Initial denaturation/activation: 95 °C, 1 min
- 40 cycles:
- 95 °C, 10–15 s

- 60 °C, 30 s
- Melting curve: 65–95 °C, 0.5 °C increments (to confirm a single amplicon).

Plate setup and replicates

- Run technical duplicates or triplicates per sample.
- Include a standard curve if quantification is required (10-fold serial dilutions of reference RNA/cDNA).

Controls per run

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

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

8.2 Interpretation and acceptance criteria

- Specific amplification: sigmoidal curve and single peak in melting analysis.
- Reproducibility: ΔC_t between replicates ≤ 0.5 .
- Negatives (NTC/Mock/blank): no specific amplification (or $C_t \gg$ limit and no specific melting peak).
- Report C_t (NS5) per sample; if a standard is included, express results as copies/ μ L or GE/mL.

Notes

- Maintain primer concentration at 0.2 μ M; if primer-dimer formation (secondary peaks) occurs, test 0.15–0.2 μ M or raise annealing temperature to 62–64 °C.
- Always use filter tips and separate areas for pre-mix and RNA handling.
- Store RNA at –80 °C; avoid more than one freeze–thaw cycle.
- For highly diluted samples, increase RNA input (up to 5 μ L), adjusting water to maintain 20 μ L final volume.

(Optional) Master Mix preparation for N reactions

Calculate per reaction and multiply by (N + 10%) to account for pipetting losses.

Mix Reaction Mix 2 $\times$ + RT Enzyme Mix 20 $\times$ + primers; aliquot. Add RNA last, on ice.

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