# ZDC (Zika, Dengue, and Chikungunya) Multiplex RT-PCR Assay

## Instruction Manual

### RT-PCR assay for the detection of Zika, dengue, and chikungunya viral RNAs

Catalog #12003818

**For research use only.**

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# Section 1
# **Introduction**

## Name and Intended Use

The ZDC Multiplex RT-PCR Assay is a real-time PCR test intended for the detection of RNA from Zika virus (ZIKV), dengue virus (DENV), and chikungunya virus (CHIKV). The results of the assay determine whether ZIKV, DENV (DEN-1, DEN-2, DEN-3, and DEN-4), and CHIKV viral RNA was detected.

The ZDC Multiplex RT-PCR Assay is intended for research use only and not for use in diagnostic procedures. The assay has been wet-lab validated for use with Bio-Rad's CFX96™ and CFX96 Touch™ Real-Time PCR Detection Systems and Thermo Fisher Scientific's Applied Biosystems 7500 Fast Dx Real-Time PCR System. The assay is also compatible with other commonly used real-time PCR instruments (see Table 1 on page 9 for system compatibility).

## Protocol Use Limitations

The ZDC Multiplex RT-PCR Assay is intended for research use only and must not be used for diagnostic purposes.

## Summary and Explanation of the Test

The ZDC Multiplex RT-PCR Assay is a real-time reverse transcription polymerase chain reaction (RT-PCR) test that enables the detection of ZIKV, DENV (DEN-1, DEN-2, DEN-3, and DEN-4 can be detected but the specific strain cannot be identified), and CHIKV viral RNAs in a variety of sample types.

Purified nucleic acids are reverse transcribed and amplified in a single step that is carried out without any extra pipetting from the operator.* The reverse transcription step takes advantage of the iScript™ Reverse Transcriptase (Bio-Rad Laboratories, included in kit) and the resulting cDNA is subsequently amplified using the iTaq™ Universal Probes One-Step Reaction Mix (Bio-Rad Laboratories, included in the kit). The reverse transcription/amplification reaction can be performed on commonly available real-time PCR systems (see Table 1 on page 9 for system compatibility).

The assay takes advantage of specific primers to first reverse transcribe RNA into cDNA and then amplify the target cDNA. The ZIKV RT-PCR assay targets the *ns4b* gene, the DENV assay targets 5'UTR sequences, and the CHIKV assay targets the *nsP2* gene. During amplification, the probes bind to the

* It is responsibility of the researcher to select and use the most appropriate methodology to obtain PCR-grade viral RNA from the sample type of interest.

ZIKV, DENV, and CHIKV target sequences. Subsequent extension of the PCR product leads to hydrolysis of the probe and separates the probe's fluorescent reporter from the quencher molecule. Subsequently, the presence of target cDNA is determined in real time by the detection of target-specific fluorescent oligonucleotide probes during amplification. A multiplexed PCR probe mix allows simultaneous amplification and detection of all three viruses (when present) and an internal control in a single reaction. In the absence of target RNA/cDNA for a given oligonucleotide probe, no corresponding fluorescence will be emitted; thus no signal will be detected.

Optional RNase P assays specifically designed for maximum compatibility with the ZDC Multiplex RT-PCR Assay are available with Cy5.5 (compatible with the CFX96, CFX96 Touch, and other qPCR systems) and TAMRA labeling and can be purchased separately. See Table 1 on page 9 for system compatibility. An RNase P positive control, ZDC Clear, is also available and can be purchased separately (catalog #12004860).

## Safety/Precautions

Good laboratory practice is compulsory and strict universal safety precautions must be taken for all activities that require the handling of samples/specimens that can be biohazardous, infectious, or contain pathogens.

The CDC provides biosafety guidance for working with Zika virus specimens at cdc.gov/zika/state-lab/index.html. Risk assessment when conducting a new laboratory test is recommended and safety precautions should be based on the outcome of the laboratory's risk assessment.

While Zika and dengue viruses have to be handled under a biosafety level 2 (BSL-2) environment, specimens that potentially harbor chikungunya virus should be handled in a BSL-3 laboratory.*

Always consider the potential danger of the specimens being tested while performing all procedures described in this document and/or the workflow for this assay. The assay has been designed to identify the presence of viral genomes. Therefore, it is assumed that the specimens contain viruses that are considered biohazardous.

Operators should be aware of the risks and safety procedures and should recognize that chikungunya produces high levels of viremia and that specimens from suspected chikungunya virus cases should be treated as potentially infectious.

* Visit cdc.gov/zika/state-lab/index.html to find CDC guidelines for state and local public health laboratories. For additional information, visit cdc.gov/biosafety/publications/bmbl5/index.htm for Biosafety in Microbiological and Biomedical Laboratories BMBL guidelines.

### General safety procedures and precautions

- This test kit should be handled only by qualified personnel trained in laboratory procedures and real-time PCR techniques and familiar with their potential hazards. Handle appropriately with the requisite Good Laboratory Practices. Wear protective clothing, including lab coat, eye/face protection, and disposable gloves (synthetic, non-latex gloves are recommended) while handling kit reagents and patient samples. Wash hands thoroughly after performing the test
- Use extreme caution when handling clinical specimens as the primary hazard associated with blood specimens from patients suspected of infection with CHIKV is inhalation of virus-containing aerosols. As coinfection with ZIKV, DENV, and CHIKV can occur, treat all specimens as potentially infectious
- Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled
- Do not pipet by mouth
- No known test method can offer complete assurance that infectious agents are absent. Therefore, all human blood derivatives, reagents, and human specimens should be handled as if capable of transmitting infectious disease. Follow recommended standard and universal precautions for blood-borne pathogens as defined by OSHA, Biosafety Level 2 guidelines from the current CDC/NIH Biosafety in Microbiological and Biomedical Laboratories, the WHO Laboratory Biosafety Manual, and/or local, regional, and national regulations
- Biological spills: human source material spills should be treated as potentially infectious
- Spills not containing acid should be immediately decontaminated, including the spill area, materials, and any contaminated surfaces or equipment, with an appropriate chemical disinfectant that is effective for the potential biohazards of the samples involved (1:10 dilution of household bleach, 70–80% ethanol or isopropanol, an iodophor such as 0.5% Wescodyne Plus, EPA registration #4959-16-52, or a phenolic) and wiped dry
- Spills containing acid should be appropriately absorbed (wiped up) or neutralized and wiped dry. The area should be decontaminated with a chemical disinfectant. Materials used to absorb the spill may require biohazardous waste disposal

**NOTE**: DO NOT PLACE SOLUTIONS CONTAINING BLEACH INTO THE AUTOCLAVE

- Dispose of all specimens and material used to perform the test as though they contain an infectious agent. Laboratory chemical or biohazardous wastes must be handled and discarded in accordance with all local, regional, and national regulations
- Complete hazard information and precautions are located in the Safety Data Sheet (SDS) available from Bio-Rad Technical Service and at **bio-rad.com**

# Section 2
# Equipment and Consumables

## Components Included in the ZDC Multiplex RT-PCR Assay

**ZDC Multiplex RT-PCR Assay - Catalog #12003818 (200 tests):**

- **iTaq™ Universal Probes One-Step Reaction Mix**, 2x, 3 x 1 mL/tube
- **iScript™ Reverse Transcriptase**, 5x, 1 x 100 µL
- **ZDC Multiplex PCR Assay Mix**, 12.5x, 1 x 440 µL
- **ZDC Internal Control**, lyophilized (resuspend in 1 mL ZDC Buffer T and dilute 1:100), 1 tube
- **ZDC Positive Control**, lyophilized (resuspend in 200 µL ZDC Buffer T), 1 tube
- **ZDC Buffer T**, 4 x 2 mL/tube
- **Nuclease-free water**, 2 x 800 µL

**Note:** The ZDC Multiplex RT-PCR Assay includes enough ZDC Buffer T to run approximately 28 positive controls. Additional ZDC Buffer T can be purchased separately, if needed (catalog #12005062).

## Equipment Required and Not Provided in the ZDC Multiplex RT-PCR Assay

- **Real-time qPCR instrument** (CFX96™ or CFX96 Touch™ Real-Time PCR Detection System (recommended) or other compatible qPCR systems. See Table 1 on page 9 for system compatibility.)
- **Vortex mixer**
- **Microcentrifuge**
- **Appropriate containers to prepare reagents**
- **Precision pipets** to deliver 0.5 µL to 1 mL of sample (accurate within ±10%) or a multichannel pipettor

## Consumables Required but Not Provided in the ZDC Multiplex RT-PCR Assay

- **1.5 mL microcentrifuge tubes for RNA sample collection**
- **PCR tubes and caps or PCR plates** (suggested plates for the CFX96 Systems: catalog #HSP9655)
- **Racks for microcentrifuge tubes**
- **Pipet tips**, sterile, nuclease-free, and aerosol resistant

- **Appropriate containers to prepare reagents**
- **Household bleach (5% to 8% sodium hypochlorite)**, which may be diluted to a minimum concentration of 10% bleach (or 0.5% sodium hypochlorite), or other RNA/DNA surface decontaminants
- **Disposable gloves**
- **Molecular-grade water**, glass distilled, RNase free

## Storage and Stability

Store the kit at –20°C. The assay is stable for six months at this temperature. Bring all reagents to 2–8°C before use. Return all reagents to –20°C immediately after use.

## Instrument Compatibility

The ZDC Multiplex RT-PCR Assay has been wet-lab validated on Bio-Rad's CFX96 and CFX96 Touch Real-Time PCR Systems and Thermo Fisher Scientific's Applied Biosystems 7500 Fast DX Real-Time PCR System. The assay is also compatible with other commonly used real-time PCR systems (Table 1). If your system is not listed, please contact Bio-Rad Technical Support in your region to check for potential compatibility. The user should select the setup appropriate for their PCR system. More details are available under **PCR Instrument Programming — Fluorescence Detectors (Dyes)** on page 17.

**Table 1. Noncomprehensive list of real-time PCR systems compatible with the ZDC Multiplex RT-PCR Assay.**

| Vendor | Real-Time PCR Instrument | 4-Plex Capability (ZIKV, DENV, CHIKV, Internal Control) | 5-Plex Capability (ZIKV, DENV, CHIKV, Internal Control, RNase P) | RNase P Compatible Assay, Catalog # | ROX Normalization |
|---|---|---|---|---|---|
| Bio-Rad | CFX96 and CFX96 Touch | Yes | Yes | 12004601 | Not required |
| Thermo Fisher Scientific | Applied Biosystems 7500 | Yes | Yes | 12004602 | Not required |
| | QuantStudio 5 | Yes | Yes | 12004601 or 12004602 | Not required |
| | QuantStudio 6 Flex | Yes | Yes | 12004602 | Not required |
| | QuantStudio 7 Flex | Yes | Yes | 12004601 or 12004602 | Not required |
| | QuantStudio 12K Flex | Yes | Yes | 12004601 or 12004602 | Not required |
| QIAGEN | Rotor-Gene | Yes | Yes | 12004601 | Not required |
| Roche | LightCycler 480 | Yes | Yes | 12004601 or 12004602 | Not required |
| | LightCycler 96 | Yes | No | N/A | Not required |

## Precautions for Users

1. Do not use the kit beyond the expiration date.
2. Do not use reagents from other manufacturers with this assay.
3. Use disposable DNase-/RNase-free pipet tips that are aerosol resistant.
4. Avoid nuclease (DNase/RNase) and microbial contamination of the specimen and components of the kit.
5. Use segregated working areas for (i) specimen preparation, (ii) reaction setup, and (iii) amplification/detection activities. Use dedicated supplies and equipment in each area. Workflow in the laboratory should always proceed in the same direction.
6. Always wear disposable gloves and change them before entering different areas.
7. To avoid contamination with amplicons, do not open the reaction tubes/plates after amplification.
8. Store positive and/or potentially positive material separately from all other kit components.

## Reagent Preparation and Storage

### Stock Reagent Preparation and Handling

Every time reagents need to be prepared, clean all working surfaces with 10% bleach followed by reagent alcohol.

### Preparing the stock internal control (IC) RNA solution

1. Resuspend the lyophilized IC RNA with 1 mL of ZDC Buffer T.
2. Dilute the resuspended IC RNA to 1:100 by combining 1 µL of resuspended IC RNA with 99 µL of ZDC Buffer T.
3. Divide the 1:100 diluted IC RNA into aliquots and label with preparation date, lot number, and expiration date. Store at –20ºC for future use. Do not freeze-thaw IC RNA more than three times.

The IC RNA solution should be added to the lysis/extraction buffer or directly into the RNA sample after resuspension into the lysis buffer. The volume of IC RNA solution that should be added depends on the elution volume and should be 1/60 of the elution volume (for example, 1 µL in 60 µL total elution volume).

### Preparing the stock positive control RNA solution

1. Resuspend the lyophilized positive control RNA with 200 µL of ZDC Buffer T.
2. Divide the resuspended positive control RNA into aliquots and label with preparation date, lot number, and expiration date. Store at –20ºC for future use. Do not freeze-thaw positive control RNA.

## Negative- and Positive-Sample Control Preparation

### Preparing the negative-sample control

To prepare the negative-sample control, add 1 µL of stock IC RNA solution from the aliquoted sample (step 3, **Preparing the stock internal control (IC) RNA solution** on page 10) to 140 µL of ZDC Buffer T. The resulting solution is the negative-sample control and should be extracted in parallel with the experimental samples and other controls.

### Preparing the positive-sample control

To prepare the positive-sample control, add 1 µL of stock positive control RNA solution from the aliquoted sample (step 2, **Preparing the stock positive control RNA solution** on page 11) to 140 µL of ZDC Buffer T. The resulting solution is the positive-sample control and should be extracted in parallel with the experimental samples and other controls.

# Section 3
# **Quality Control**

Real-time RT-PCR is a sensitive procedure and should be conducted following strict quality control and quality assurance procedures. Following these guidelines will help minimize the chance of false-positive and false-negative results.

## General Considerations

- Operators assigned to use this assay should be trained professionals and familiar with the protocol and instruments used
- Use segregated working areas for (i) specimen preparation, (ii) reaction setup, and (iii) amplification/detection activities. Use dedicated supplies and equipment in each area. Workflow in the laboratory should always proceed in the same direction
- The workflow should always follow one direction from RNA extraction and reagent preparation that must be performed in a clean area to RT-PCR reaction setup that should be performed in a dirty area. This approach will minimize the risk of contamination of the clinical samples by amplified nucleic acids
- Use different pairs of powder-free gloves for the work carried out in the clean area and for the procedures conducted in the dirty area
- Do not use reagents from other manufacturers with this assay (unless specified in this document)
- Use disposable DNase-/RNase-free pipet tips that are aerosol resistant
- Avoid nuclease (DNase/RNase) and microbial contamination of the specimens and kit components
- To avoid contamination with amplicons, do not open the reaction tubes/plates after amplification
- Store positive and/or potentially positive material separately from all other kit components

## Assay Control

### Internal control

Bio-Rad's ZDC Multiplex RT-PCR Assay includes a ZDC Internal Control. The ZDC Internal Control is composed of a known synthetic RNA sequence that has no homology with any human sequence. It is added either to the RNA lysis/extraction buffer or to the RNA sample once resuspended into

the lysis buffer. The internal control will then be purified, reverse transcribed, and amplified together with any viral RNA present in the sample. This control provides information regarding the quality of the extraction and the potential carryover of inhibitors. The primers and probe for detection of the internal control by RT-PCR are included in the ZDC Multiplex RT-PCR Assay Mix. The internal control should also be added to the positive controls.

### Positive control

The ZDC Positive Control includes three in vitro transcribed RNA molecules carrying sequences that are homologous to Zika (ZIKV), dengue (DEN-1) (DENV), and chikungunya (CHIKV) viruses and are targeted by the ZDC Multiplex RT-PCR Assay. These synthetic RNAs should be spiked into ZDC Buffer T or ZDC Clear (catalog #12004860) as described in **Section 2 — Equipment and Consumables — Reagent Preparation and Storage**. This control should be extracted and run in parallel any time a test is conducted and a reaction is prepared.

**Note**: Positive controls represent a source of cross-contamination. Precautions should be taken to prevent and minimize the risk. To reduce the risk of cross-contamination, refer to the **General Considerations** in this section.

### No template control (NTC)

NTC reactions include PCR-grade water (provided with the ZDC Multiplex RT-PCR Assay) in place of specimen RNA. This control should be included for each reaction mixture in each run. This control provides important information about the presence of potential contaminants and functionality of the assay/environmental contaminations that result in false-positive results.

### Negative control

The negative control is composed of internal control (IC) RNA–spiked ZDC Buffer T (see **Preparing the negative-sample control** on page 11). This control should be run in parallel with the experimental samples each time a run is set up. It provides information about the presence of potential environmental contaminants and the eventual malfunctioning of primers/probes.

### Sample control (optional)

RNase P assays (catalog #12004601 or 12004602) can be used as sample controls together with ZDC Clear (catalog #12004860). This control can be included in the ZDC RT-PCR Assay as an additional target or run separately. ZDC Clear will serve as a positive control for RNase P and a negative control for viral targets. This control provides information about the quality of the starting material and the extraction procedure.

# Section 4
# Extraction of Nucleic Acids

## Notes on Extraction

- The ZDC Internal Control should be added in each extraction run as an internal control
- Never add carrier RNA (cRNA), internal control (IC) RNA, or positive control RNA directly to the sample as enzymes potentially present in the specimen could degrade the RNA
- cRNA is critical to maintaining the extraction efficiency and stability of extracted RNA
- Elimination of any carryover ethanol from the extraction to the PCR step is crucial; ethanol will strongly inhibit real-time PCR

# Section 5
# RT-PCR Assay

## Preliminary Statement

1. Assay controls should be run with all test samples. Assay validity is determined by the controls run with each assay.
2. Each individual run should include three control reactions:
   - 1 negative control (negative for Zika (ZIKV), dengue (DENV), and chikungunya (CHIKV)): ZDC Clear + internal control (IC) RNA or ZDC Buffer T + IC RNA
   - 1 positive control (positive for ZIKV, DENV, and CHIKV): positive control + ZDC Buffer T
   - 1 no template control ($H_2O$)

## RT-PCR Assay Procedure

### Preparation of the PCR Master Mix

1. Thaw and mix the reagents carefully by pipetting up and down or flicking the tubes gently. Briefly spin reagent vials in a microcentrifuge before opening. Store in a cooling block prior to use and limit exposure to light.
2. Prepare the PCR master mix in a 1.5 mL microcentrifuge tube according to Table 2. Store in a cooling block and limit exposure to light until ready to use.

**Table 2. Preparation of the reaction mix.**

| ZDC Assay Reaction Mix | 1x | 10x |
|---|---|---|
| 2x iTaq™ Universal Probes One-Step Reaction Mix, µL | 12.5 | 125 |
| iScript™ Reverse Transcriptase, µL | 0.6 | 6 |
| ZDC Multiplex PCR Assay Mix (12.5x), µL | 2 | 20 |
| Template, µL | (5) | –* |
| Water (molecular grade), µL | 4.9 | 49 |
| Total, µL | 25 | 200 |

* 5 µL of sample will be added to each tube separately.

**Note**: Prepare 10% excess volume of master mix to ensure accurate pipetting.

### Reaction Setup

1. Pipet 20 µL of the master mix into each well of an optical 96-well reaction plate, as required, or into an optical reaction tube.
2. Add 5 µL of sample (eluate from the nucleic acid extraction) or 5 µL of a control (positive, negative, and NTC control). At least one positive, one negative, and one NTC control must be included in each run.
3. Seal the 96-well reaction plate with an optical adhesive film or the reaction tubes with appropriate lids.
4. Thoroughly mix the samples and controls with the master mix by vortexing for 15 sec.
5. Centrifuge the 96-well reaction plate in a centrifuge with a microtiter plate rotor for 30 sec at approximately 1,000 x g (~3,000 rpm).

### PCR Instrument Programming

Details regarding the setup and programming of Bio-Rad's CFX96™ or CFX96 Touch™ Real-Time PCR Detection Systems or other real-time PCR instruments can be found in the system instruction manual or by contacting the manufacturer. Apply the following parameters for the multiplex RT-PCR assay.

#### 1. Settings

| Settings | |
|---|---|
| Reaction volume | 25 µL |
| Ramp rate | Default |
| Passive reference | None |

**Note**: Bio-Rad's CFX96 and CFX96 Touch Systems do not require any passive reference and the option is not available. For systems that have an option for passive reference (for example, the Applied Biosystems 7500 Fast Dx instrument), ensure that the passive reference option is switched off.

## 2. Fluorescence Detectors (Dyes)

| Target | CFX96 or CFX96 Touch System Fluorophore/Channel |
|---|---|
| ZIKV | FAM |
| CHIKV | HEX |
| DENV | Texas Red |
| Internal control (IC) | Cy5 |
| RNase P (optional)* | Cy5.5** |

* To be bought separately; not included with the ZDC Multiplex RT-PCR Assay.

** The RNase P assay is also available with TAMRA labeling. See Table 1 on page 9 for system compatibility.

## 3. Temperature Profile and Dye Acquisition

| | Stage | Cycle Repeats | Acquisition | Temperature, °C | Time |
|---|---|---|---|---|---|
| Reverse Transcription | Hold | 1 | – | 50 | 15 min |
| Denaturation | Hold | 1 | – | 94 | 2 min |
| Amplification | Cycling | 45 | – | 94 | 15 sec |
| | | | – | 55 | 40 sec |
| | | | • | 68 | 30 sec |

# Section 6
# **Interpretation of Results**

- All test controls should be examined prior to interpretation of sample results. Table 3 provides a summary of the different recommended controls as well as their expected results
- A Cq value will be assigned for each amplification reaction occurring in a reaction well. The Cq value represents the cycle at which the fluorescence detected for a specific channel exceeds the set threshold. The Cq resulting from interpolation of the fluorescence curve and the threshold can be the result of fluorescence generated by a specific amplification of the desired target or it can be a nonspecific fluorescence signal
- Amplification plots should be analyzed for all samples. A positive amplification is represented by an exponential increase in fluorescence over the background. If the raw data (baseline not subtracted/normalized) does not exhibit any exponential increase of fluorescence, the target has probably not been amplified. If the amplification plot does not exhibit an exponential fluorescence increase crossing the threshold, or if the baseline-not-subtracted plot of Bio-Rad's CFX96™ or CFX96 Touch™ Real-Time PCR Detection System does not exhibit an increase in fluorescence, the target molecule has probably not been amplified
- If the analysis software assigns a Cq value to the amplification curve but the amplification curve does not exhibit a sustained exponential growth (followed by a plateau) that crosses the set threshold, the sample should be considered undetermined
- An internal control should be included in every sample
- Cq values for the internal control and Zika (ZIKV), dengue (DENV), and chikungunya (CHIKV) viruses could vary depending on sample type and quality. Proper experimental procedure should be followed to accurately define the correct setup conditions for different specimens

## Test Assessment

**Table 3. Test validity determination — summary of expected results.**

| Control Type | Control Name | Purpose of Control | ZIKV | DENV | CHIKV | Internal Control |
|---|---|---|---|---|---|---|
| ZDC Positive Control | ZIKV + DENV + CHIKV + IC | Quality of the extraction procedure; environmental contaminants; determination of primers/probes malfunction; RT | + | + | + | + |
| No Template Control | Water | Environmental contaminants; primers/probes malfunctioning | — | — | — | — |
| Negative Control | ZIKV + DENV + CHIKV + IC | Quality of the extraction procedure; environmental contaminants; determination of primers/probes malfunction | — | — | — | + |

**For a test to be reliable, the controls should meet the following criteria:**

a. **The negative control must be negative**, showing no fluorescent signal above the threshold in the ZIKV (FAM), DENV (Texas Red), and CHIKV (HEX) channels. Cq cut-off values for the internal control (Cy5) should be determined by the operator based on experimental design.

b. **The positive control must be positive**, showing a fluorescent signal in the ZIKV (FAM), DENV (Texas Red), CHIKV (HEX), and internal control (Cy5) channels. Cq cut-off values should be determined by the operator based on experimental design.

c. **The internal control must be positive for all samples, the positive control, and the negative control (except NTC)**. Cq cut-off values for the internal control (Cy5) should be determined by the operator based on experimental design.

# Section 7
# Assay Limitations

**This test is intended for research use only and not for use in diagnostic procedures.**

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