RNA isolation and RT-qPCR for Dengue, Chikungunya and Zika Viruses

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We use this protocol and it's working

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ABSTRACT

This protocol is for dried blood spot (DBS) sample collection, the extraction of viral RNA from dried blood spots, and the following RT-qPCR setup for the detection of Dengue, Chikungunya, and Zika viruses.

MATERIALS

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- double distilled water (ddH2O) **Contributed by users**
- QIAamp Viral RNA Mini Kit **Qiagen Catalog #52904**
- Bunsen burner **Contributed by users**
- Quantinova SYBR Green RT-PCR Kit **Qiagen Catalog #208154**
- 6-mm Paper Punch **Contributed by users**
- Applied Biosystems StepOnePlus™ Real-Time PCR System **Thermo Fisher Scientific Catalog #4376600**
- Whatman® 903 protein saver cards **Sigma Aldrich Catalog #WHA10531018**

BEFORE START INSTRUCTIONS

During RT-qPCR preparation, it is important to avoid DNA cross-contamination. Use an area exclusively for RNA work.

Clean the working area and all equipment with 10% bleach.

Dispose of contaminated sharps and waste appropriately

Blood Sample Collection

1 1. Wear gloves and label the filter paper to be used before collecting DBS.

2. Clean the finger from which sample will be collected with 75% alcohol.

3. Fingerprick with a small lancet after alcohol dries off. Apply gentle pressure to the finger and allow a large drop of to collect at the puncture site.

4. Remember NOT to press the filter paper against the puncture wound, rather allow the blood to drop and saturate the full circle. Avoid smering the blood spot.

5. After the blood spots are completely on the filter paper, allow it to dry before placing it in a sealed plastic bag.

6. Store the filter paper in the sealed plastic bag with a desiccant package and then store it in closed box. Avoid exposure to the sun.

RNA Viral Extraction

2 1. Use the paper puncher to punch a 6-mm punch in the dried blood spot. Sterilize the paper puncher with fire and make a clean punch after each use to avoid cross-contamination.

2. Extract Viral RNA utilizing the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions..

RT-qPCR

3 3. Prepare the RT-qPCR master mix using the Quantinova SYBR Green RT-PCR Kit for the three arboviruses. Three final volumes of 25 µl of PCR mixes contained 5 µL of RNA extract with individual DENV, CHIKV, or ZIKV primer pairs at a concentration of 0.3 µM, 0.3 µM, and 0.4 µM, respectively.

Primers

Primer	Sequence 5' to 3'	Gen Bank Reference No.
DENV-F	CAA TAT GCT GAA ACG CGA GAG AAA	AF038403
DENV-R1-3	CCC CAT CTA ACC AAT ATT CCT GCT	AF180817, AF038403, M93130
DENV-R4	CCC CAT CTG TTC AGT ATC CCT GCT	M14931
CHIKV-F	AAG CTY CGC GTC CTT TAC CAA G	EU192142, EU192143
CHIKV-R	CCA AAT TGT CCY GGT CTT CCT	EU192142, EU192143
ZIKV-F	GCA ACA TGG CGG AGG TAA GAT	KU321639
ZIKV-R	GCT CTY GGT GAA TTR GGC GT	KU321639

Master Mix

Dengue		Chikungunya		Zika	
Master Mix	µL	Master Mix	µL	Master Mix	µL
RNA Sample	5.0	RNA Sample	5.0	RNA Sample	5.0
ddH20	3.75	ddH20	4.5	ddH20	4.0
SYBR green	12.5	SYBR green	12.5	SYBR green	12.5
RT Mix	0.25	RT Mix	0.25	RT Mix	0.25

	Primer-F (0.3 µM)	0.75	Primer-F (0.3 µM)	0.75	Primer-F (0.4 µM))	1.0
	Primer-R1 (0.3 µM)	0.75	Primer-R (0.3 µM)	0.75	Primer-R (0.4 µM)	1.0
	Primer-R2 (0.3 µM)	0.75	Rox	1.25	Rox	1.25
	Rox	1.25				
	Total	25		25		25

4. Process the RT-qPCR in the Applied Biosystems StepOnePlus™ Real-Time PCR System with the following conditions:

	1. RT	**1 cycle**
	50 °C for 30 min	
	2. Pre-incubation	**1 cycle**
	95 °C for 15 min	
	3. 3-step amplification	**45 cycles**
	95 °C for 15 s	
	55 °C for 30 s	
	72 °C for 20 s	
	77 °C for 20 s	
	4. Melting	**1 cycle**
	95 °C for 60 s	increase in temperature (1°C/30 s) to 90 °C
	60 °C for 30 s	
	97 °C for 1 s	

5. A sample was considered positive if the fluorescence curve crossed the threshold line within 40 cycles (Cq<40); and if the melting curve for DENV was around 80.25°C for DENV-1, 81.75°C for DENV-2, 80.,5°C for DENV-3 and DENV-4 for 83°C, for CHIKV and ZIKV was between 81°C and 82°C. Send positive samples for sequencing.