Real-time Reverse Transcription Polymerase Chain Reaction (RT- qPCR)

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Abstract

For investigate the presence of YFV genome by quantitative PCR assays in the hydrolysis probe detection format. The target is the 5'-noncoding region (5'-NC) of the YFV genome (Domingo et al., 2012).

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Materials

- Microcentrifuge Tubes by Contributed by users
- Filter Tips by Contributed by users

  Nuclease-Free Water, 1000ml P1199 by Promega

  GoTaq® Probe 1-StepRT-qPCR System A6120 by Promega
- Primer by Contributed by users
- Probe by Contributed by users
- Micropipettors by Contributed by users
- Real time PCR instrument by Contributed by users
- PCR tubes (for qPCR) by Contributed by users
- PCR plate (for qPCR) by Contributed by users
- adhesive plate seal by Contributed by users

Protocol

Step 1.
Each sample is tested at least in duplicate, or triplicate.

Step 2.
The final reaction volume in this protocol is 20µl.

Step 3.
Estimate the appropriate amount of each reagent for each test/sample.

Step 4.
Determine the number of reactions to be set up, including negative control from the RNA extraction, for the non-template control and for the positive control reactions.

Step 5.
Add 1 or 2 reactions to this number to compensate for pipetting error.

Step 6.

Prepare the reaction mix (minus the RNA template) by combining the GoTaq® Probe qPCR Master Mix with dUTP, GoScript™ RT Mix for 1-Step RT-qPCR, primers, hydrolysis probe and Nuclease-Free Water as described below. Vortex briefly to mix.

Component	Volume	Concentration
GoTaq® Probe qPCR Master Mix with dUTP*	10 µL	1X
GoScript™ RT Mix for 1-Step RT-qPCR	0,4 µL	1X
Forward primer 20X (Domingos et al, 2012)	1,0 µL	200 nM-1µM
Reverse primer 20X (Domingos et al, 2012)	1,0 µL	200 nM-1µM
Hydrolysis probe (Domingos et al, 2012)	0,5 µL	100-300nM
RNA temlate	5,0 µL	10pg-1µg
Nuclease-Free Water	To final volume of 20 µL	

*The GoTaq® Probe qPCR Master Mix included in the GoTaq® Probe 1-Step RT-qPCR System is formulated with dUTP. When dUTP is incorporated into the amplification products, the amplicons are susceptible to degradation by uracilDNA glycosylase (UNG); this allows you to incorporate UNG into subsequent reactions to control possible carryover contamination.

Step 7.
Add the appropriate volume of reaction mix (without the RNA template) to each PCR tube or well of an optical grade PCR plate.

Step 8.
Add the RNA template (or water for the no-template control reactions) to the appropriate wells of the reaction plate.

Step 9.
Seal the tubes or optical plate; centrifuge briefly to collect the contents of the wells at the bottom. Protect from extended light exposure or elevated temperatures before cycling. The samples are ready for thermal cycling.

Step 10.

Run the cycling parameters below:

Step	Cycles	Temperature	Time
Reverse transcription	1	45°C	15 minutes
Reverse transcriptase inactivation and GoTaq® DNA Polymerase activation	1	95°C	2 minutes
Denaturation	40	95°C	15 seconds

Annealing and extension	60°C	1 minutes

Step 11.
When the cycling ends, analyze the results according to the manufacturer's instructions.

Step 12.
RNA extraction control and non-template controls must be negative and positive control must be positive.

Step 13.
To be considered positive a sample must present at least two replicates with amplification equal of bellow the cycle threshold of 37.