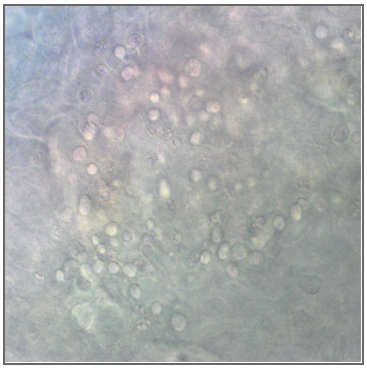

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Protocol status: Working
We use this protocol and it's working

Protocol for quantification of BdDV-1 mycovirus of Batrachochytrium dendrobatidis by qPCR

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ABSTRACT

This protocol is used for quantifying the amount of the DNA mycovirus BdDV-1 in DNA extracts or cDNA libraries. This virus is associated with the fungus Batrachochytrium dendrobatidis (Bd), and it is known to be endogenized in the genome of a large number of Bd strains. The assay uses real time quantitative PCR with Taqman probes. Setup of the reaction is typically done in a biosafety cabinet. This protocol has been optimized on a QuantStudio 3 (Applied Biosystems).

ATTACHMENTS

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PROTOCOL integer ID: 80313

Keywords: BdDV-1 qPCR

GUIDELINES

Notes:

- BdV F: CCTGAGTACCCTGATCACAATGT
- BdV R: GGGTCATTGGTCGTATCTTCA
- BdV Probe: MBGNFQ-CCATGGTGGCGTTCT-NED

Standards for viral copy number were created by cloning PCR products from BdV F and BdV R primers into pCR 2.1-TOPO vector using the TOPO TA cloning kit. Plasmids from transformed E. coli colonies with successful insert were extracted using the Zyppy Plasmid Miniprep kit. DNA of plasmid extracts was quantified with a Qubit. Plasmid copy numbers were estimated using the formula:

Number of copies per uL = [(g/uL) / plasmid length in bp * 650)] * ($6.022 * 10^{23}$)

Extracts were then diluted to a final concentration of 10^6 viral copies per total 35uL volume for qPCR standards.

MATERIALS

Materials:

- 96 well optical qPCR plate (Applied Biosystems 4306737)
- p1000, p200, p20, p10 pipettes
- Barrier tips for all pipettes (recommend using a new unopened box of 10uL tips)
- Optical adhesive sealing film (Applied Biosystems 4311971)
- Tabletop centrifuge (for spinning down samples and reagents)
- Large centrifuge (for spinning down 96 well plate)
- PCR water
- Radiant Probe Lo-ROX qPCR Kit (Alkali Scientific QP9005)
- BdV F and BdV R Primers ([M] 18 micromolar (µM))
- BdV Probe ([M] 5 micromolar (µM))—**light sensitive**, tubes should be wrapped in aluminum foil when not in use
- BSA (400 ng/µl)
- 10^6 BdDV-1 standard

MicroAmp™ Optical 96-Well Reaction Plate with Barcode **Thermo Fisher Catalog #4306737**

ABI prism optical adhesive covers **Thermo Fisher Scientific Catalog #4311971**

Radiant™ Probe Lo-ROX qPCR Kits **Alkali Scientific Catalog #QP9005**

Protocol:

15m 41s

1 Wipe down pipettes with 70% ethanol and place in the biosafety hood with tips and 96 well plate. UV for at least 00:15:00 (longer is fine). 15m

2 After setting up hood to UV, prepare standard dilutions on the bench using barrier tips. Fill 6 1.5uL microcentrifuge tubes with 90 µL of PCR water. Label the tubes 10^5-10^0.

2.1 Add 10 µL of the 10^6 virus standard to the 10^5 tube, pipetting up and down several times to mix.

2.2 Repeat this, adding 10 µL from the 10^5 tube to the 10^4 tube and so on. You should have 7 standards total, 10^6-10^0.

3 Prepare the master mix in the biosafety hood. All reagents can be thawed quickly on the bench, flicked to mix, and briefly spun down on a tabletop centrifuge before preparation. All the reagents and master mix should be kept On ice during the preparation. Volumes per well:

	A	B
	Radiant Probe Lo-ROX qPCR Kit	12.5 uL
	PCR water	2.75 uL
	BdV F primer (18 uM)	1.25 uL
	BdV R primer (18 uM)	1.25 uL
	BdV probe (5 uM) –protect from light when not in use	1.25 uL
	BSA (400 ng/uL)	1 uL

Multiply above values by the total number of wells on the plate that will be used plus an additional 2 wells for pipette error. Standards and negative control should be run in triplicate, samples should be run in duplicate. If total master mix volume exceeds 1.5 mL, divide total volume in 2 and prepare master mix in 2 identical 1.5mL tubes. Add the appropriate amount of each reagent to each 1.5mL tube, working On ice with barrier tips. Once all the reagents have been added, flick the tubes to mix and briefly spin down. Return the tubes to the ice.

4 Add master mix to the 96 well plate. Put the plate on a 96 well rack and place it On ice. Add 20 µL of master mix to each well on the plate that will be used. A single barrier tip can be used for all the wells.

5 Add standards and samples to the 96 well plate. Standard tubes (prepped in step 2) and samples should be thawed, flicked to mix, and briefly spun down on a tabletop centrifuge. Add 5 µL of each standard or sample to the appropriate well, using a new 10uL tip for each well. If you are using a new box of tips, you can use the placement of tips in the box to help keep track of which

wells have been loaded. Negative control wells should be loaded with PCR water. Example plate setup below (where RAC### are experimental sample numbers):

	A	B	C	D	E	F	G	H	I	J	K	L	M
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
	A	10^6	10^6	10^6	RAC70	RAC70	RAC84	RAC84	RAC108	RAC108	RAC123	RAC123	RAC136
	B	10^5	10^5	10^5	RAC71	RAC71	RAC85	RAC85	RAC110	RAC110	RAC126	RAC126	RAC136
	C	10^4	10^4	10^4	RAC72	RAC72	RAC86	RAC86	RAC111	RAC111	RAC127	RAC127	RAC137
	D	10^3	10^3	10^3	RAC73	RAC73	RAC87	RAC87	RAC117	RAC117	RAC131	RAC131	RAC137
	E	10^2	10^2	10^2	RAC80	RAC80	RAC88	RAC88	RAC118	RAC118	RAC132	RAC132	RAC138
	F	10^1	10^1	10^1	RAC81	RAC81	RAC89	RAC89	RAC120	RAC120	RAC133	RAC133	RAC138
	G	10^0	10^0	10^0	RAC82	RAC82	RAC101	RAC101	RAC121	RAC121	RAC134	RAC134	RAC139
	H	negative	negative	negative	RAC83	RAC83	RAC106	RAC106	RAC122	RAC122	RAC135	RAC135	RAC139

6 Once all samples have been added to the plate, seal the plate with optical adhesive sealing film. Make sure that the edges and corners are well sealed. Using the corner of a plastic sealing tool to press down the edges and corners is helpful.

7 Spin down the entire plate in a centrifuge briefly to make sure the liquid is at the bottom of the wells. Try to remove any bubbles be gently tapping the plate against the bench and spinning again.

8 Set up the qPCR machine for the run. Make sure that the plate is On ice while you are doing this step (it can also be done ahead of time).

8.1 Open up the QuantStudio software. Name the experiment. Set Run Mode to “Fast”.

8.2 Make sure total well volume is set to 25 µL. Run conditions are: 41s

1. 95 °C for 00:00:20 (set ramp up to 3.96)
2. 50 cycles of: 95 °C for 00:00:01 (set ramp up to 3.96) then 60 °C for 00:00:20 (ramp down to 3.02).

8.3 Input the names for your wells into the software. This can be done manually or by copying and pasting the sample names in a list format from excel, where wells are labeled 1-96 and sample names are listed in order of A1-A12, then B1-12, etc.

8.4 Mark all of the standard wells as standards in the software. Input the correct quantities for each of the standards (e.g., the quantity for the 10^6 sample should be input as 1000000).

8.5 Label the negative control wells as negatives and the rest of the occupied wells as sample wells (or unknowns).

9 Place the 96 well plate into the machine (making sure it is in the correct orientation) and start the run!

10 At the end of the run, check the standards to make sure that the replicates reasonably overlap with one another and that there is no amplification in the negative controls. It is not unusual to have outliers or no amplification in some of the lower dilutions. Check the standard curve. Efficiency should be between 90-110%, R2 should be as close to 1 as possible, and error should be as close to 0 as possible. If necessary, outlier standard replicates can be removed in the software to change these values and the samples can be reanalyzed.

11 Export your data as an .xls file. This will contain the quantities for each sample, including the mean and SD of the replicates.