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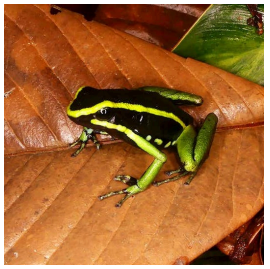
qPCR assay for detecting *Batrachochytrium dendrobatidis*



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[1]eDNA solutions; [2]Water Circle

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

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Abstract

The fungus *Batrachochytrium dendrobatidis* (Bd) was first detected in Norway in 2017, and thus indicate the arrival of an invasive black-listed species in the country.
Here we report the details of real time PCR assay which was used to screen for *B. dendrobatidis* from water samples collected from different locations in Norway.

Guidelines

Laboratory work space and equipment were sterilized by UV-light and DNase solution and 70% ethanol. Filter pipet tips were used in all steps of the laboratory work.
Negative controls of DNase/RNase free water were used in each qPCR assay.

Materials

MATERIALS

- UltraPure™ DEPC-treated Water **Thermo Fisher Catalog #**10813012
- SsoAdvanced Universal Probes Supermix **Bio-Rad Laboratories Catalog #**172-5280
- Batrachochytrium dendrobatidis 5.8S ribosomal RNA (5.8S) genesig Standard Kit

Safety warnings

Handling high concentration of positive controls was performed in a post-PCR room which is physically separated from the pre-PCR room to avoid contamination.
Always add your samples first and seal them before adding the serial dilutions of positive control (standard) at the end.

DNA extraction

1 DNA extraction was performed using Qiagen DNeasy power water sterivex kit. The quality of the extracted DNA was estimated using Nanodrop.

Qiagen DNeasy power water sterivex kit:
https://www.qiagen.com/se/resources/resourcedetail?id=c5fe7d5f-070a-4ebe-ac04-4bbf05a13e91&lang=en

2 **Internal DNA extraction control**

When performing DNA extraction, it is often advantageous to have an exogenous source of DNA template that is spiked into the lysis buffer. This control DNA is then co-purified with the sample DNA and can be detected as a positive control for the extraction process. Successful co-purification and qPCR for the control DNA also indicates that PCR inhibitors are not present at a high concentration.

2.1 **Internal control**

4 µL of the internal control were mixed with lysis buffer as given in the extraction protocol.
Purification and amplification of the internal control DNA was tested as well as the presence of PCR inhibitors.

The primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers.

Amplification of the control DNA does not interfere with the detection of the *B. dendrobatidis* target DNA even when present at low copy number.

3 **Real Time PCR**

A *B.dendrobatidis* specific primer and probe mix is provided by Primerdesign Ltd which was detected through the FAM channel. The primer and probe mix provided exploits the so-called TaqMan® principle.

During PCR amplification, forward and reverse primers hybridize to the *B.dendrobatidis* DNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labeled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of qPCR platforms.

	Component	Resuspend in water
	B.dendrobatidis primer/probe mix (BROWN)	165 µl
	Internal extraction control primer/probe mix (BROWN)	165 µl

4 Positive and internal controls

Safety information

* This component contains high copy number template and is a VERY significant contamination risk.
It must be opened and handled in a separate laboratory environment, away from the other components.

Resuspend the internal control template and positive control template in the template preparation buffer supplied, according to the table below:

	Component (heat-sealed foil)	Resuspend in template preparation buffer
	Internal extraction control DNA (BLUE)	600 µl
	B.dendrobatidis Positive Control Template (RED) *	500 µl

To ensure complete resuspension, vortex each tube thoroughly.

Preparation of standard curve dilution series

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1) Pipette 90 µl of template preparation buffer into 8 tubes and label them.
2) Pipette 10 µl of Positive Control Template (RED) into tube 2
3) Vortex thoroughly
4) Change pipette tip and pipette 10 µl from tube 2 into tube 3
5) Vortex thoroughly

Repeat steps 4 and 5 to complete the dilution series

	Positive control	Copy number
	Tube 1 Positive control (RED)	1e6 per 5 µl
	Tube 2	1e5 per 5 µl
	Tube 3	1e4 per 5µl
	Tube 4	1 e3 per 5 µl
	Tube 5	1e2 per 5 µl

	Tube 6	1e1 per 5 µl
	Tube 6.5	5 per 5 µl
	Tube 7	1e-1per 5 µl
	Tube 8	1e-2 per 5 µl

A standard with very low copy number (theoretically 0.1 cells per PCR reaction) was prepared to determine limit of detection of this assay.

PCR program for BD detection

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	Component	µl	µl
	SsoAdvanced Universal Probes Supermix	10	5
	B.dendrobatidis primer/probe mix (BROWN)	1	0,5
	RNase/DNase free water (WHITE)	4	1,5
	Final Volume	15	7

Pipette 5 µl DNA template if the total PCR mixture is 20 µl or 3 µl of DNA template if the total PCR mixture is 10 µl.
For negative control wells, we used 5 or 3 µl of RNase/DNase free water.

PCR program

7 02:30:00

		Step	Time	Temp (C)
		Enzyme activation	2 min	95
	Cycling x50	Denaturation	10 s	95
		DATA COLLECTION *	1min	60

* Fluorogenic data should be collected during this step through the FAM channel.

PCR for internal control

8

*

	Component Volume	Volume (µl)
	SsoAdvanced Universal Probes Supermix	10
	Internal extraction control primer/probe mix (BROWN)	1
	RNase/DNase free water (WHITE)	3
	Final Volume	15

Same above PCR program was used but data is collected through VIC channel.

Pipette 5 µl of DNA template to reach 20 µl final volume in each well.

For negative control wells use 5 of RNase/DNase free water.

9 QPCR was performed in BioRad qPCR machine CFX96.
Analysis of the results was done by CFX maestro software
https://www.bio-rad.com/en-se/product/cfx-maestro-software-for-cfx-real-time-pcr-instruments?ID=OKZP7E15 02:30:00

Interpretation of results

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Expected result

Quantitation cycle (Cq)
Positive control template, $1e^6$ per 5 µl, is expected to amplify between Cq 16 and 23.
Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.
Where the test sample is positive and the negative control is positive with a Cq > 35, the sample must be reinterpreted based on the relative signal strength of the two results:

Internal PCR control
The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of DNA added to the PCR reaction and the individual machine settings. Cq values of 28±3 are within the normal range.
When amplifying a *B. dendrobatidis* sample with a high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

	Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
	≤ 30	+/ -	+	-	POSITIVE QUANTITATIVE RESULT calculate copy number
	> 30	+	+	-	POSITIVE QUANTITATIVE RESULT calculate copy number
	> 30	-	+	-	POSITIVE QUALITATIVE RESULT do not report copy number as this may be due

					to poor sample extraction
	-	+	+	-	NEGATIVE RESULT
	+ /-	+/ -	+	≤ 35	EXPERIMENT FAILED due to test contamination
	+ /-	+/ -	+	> 35	*
	-	-	+	-	SAMPLE PREPARATION FAILED
	+ /-	+/ -	-	+ /-	EXPERIMENT FAILED