Sylphium
molecular ecology

Ondatra zibethicus
qPCR detection kit

With eDNA qPCR hot start mix

#SYL119 validation report

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For general laboratory and research use only.

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1. Validation report qPCR *Ondatra zibethicus* detection kit

1.1. In silico validation

Forward primer	Forward primer	Reverse primer	Probe
Length (bp)	24	22	35
GC %	45	32	32
Stability	2.4	1.8	73
T_M (°C)	61	62	CytB (mtDNA)
Target region	CytB (mtDNA)	CytB (mtDNA)	No
Dimer	No	No	No
Run	No	No	35
Database hit	*Ondatra zibethicus*	*Ondatra zibethicus*	
Fluorescence label			FAM

Table 1. *In silico* validation of probe and forward and reverse primers.

PCR product size (bp)	143
Combined dimer formation	No
In silico PCR on Genbank	*Ondatra zibethicus, Sitta yunnanensis, Cyornis ruficauda, Atlapetes albinucha, Niltava macgrigoriae*
Date of In silico PCR	June 2022

Table 2. Combined primers and probe *in silico* validation.

1.2. Experimental validation

All validation experiments were conducted using the Sylphium qPCR mix and analyzed with the BIO-RAD CFX96 Touch Real-Time PCR Detection System.

1.2.1. Gradient PCR

Influence of the annealing temperature on the performance and specificity of the primer set was tested with a temperature gradient between 50.9°C to 70.0°C in eight steps. Optimal temperature and the temperature range in which the test can perform was determined.

Results:

The expected product was formed between 50.0°C and 63.6°C. No nonspecific additional fragments were formed. No primer dimer were formed at any tested temperature. The optimal annealing temperature was between 50.0°C and 63.6°C (**Table 3**).

Annealing temp.	50.0°C	51.9°C	56.4°C	58.8°C	61.2°C	63.6°C	68.1°C	70.0°C
Expected fragment	Strong	Strong	Strong	Strong	Strong	Strong	No	No
Primer dimer	No	No	No	No	No	No	No	No
Additional fragments	No	No	No	No	No	No	No	No

Table 3. Temperature gradient PCR on DNA of *Ondatra zibethicus.*

1.2.2. Detection limit, fluorescence output signal and efficiency

Standard solutions with 10^5, 10^4, 10^3, 10^2, 10 and 0 target copies per 2 µL were analyzed to determine the detection limit at optimal primer/probe concentrations. Fluorescent output signals were compared with the background to have at least a 100-fold increase in fluorescence signal. Reaction condition were adjusted if the fluorescence signal was too low in contrast to the background noise. Based on the slope of the standard curve, the efficiency of the primer/probe set was determined. The efficiency should be between 90% and 110%. Limits of quantification and detection were determined on basis of this efficiency limitations.

Results:

Limit of detection (LDqPCR) for this kit was determined at 1 copy per reaction. Limit of quantification (LQqPCR) was determined at 10 copies per reaction. The fluorescence output signal was at least 100 (5 for positive sample, 0,05 for a negative sample) times stronger than the background signal (**Figure 1&2**, **Table 4&5**).

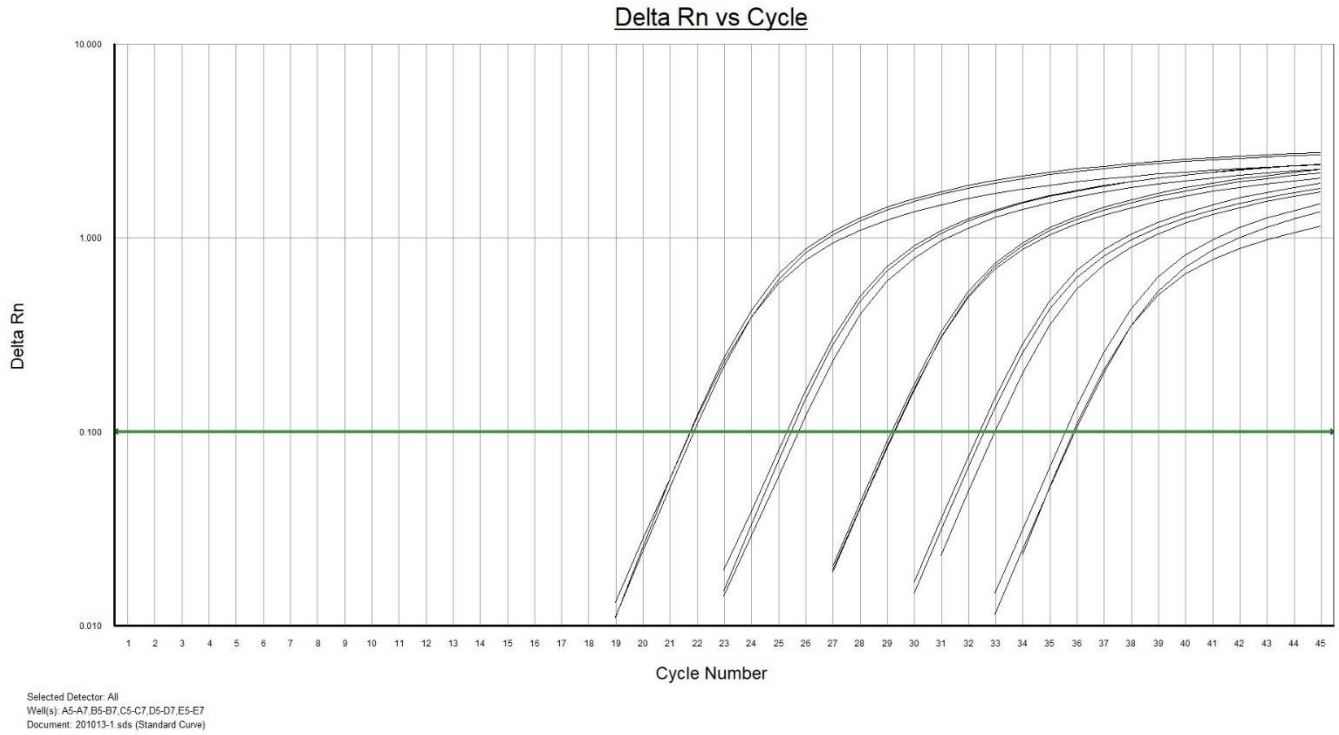

Figure 1. Obtained fluorescent signal at optimal primer/probe concentration at a annealing temperature of 65°C.

target DNA copy	Target detected	CT value	Standard deviation
0	No	-	-
1	Yes	n.a.	n.a.
10	Yes	35.8	0.2
10^2	Yes	32.6	0.3
10^3	Yes	29.2	0.0
10^4	Yes	25.4	0.2
10^5	Yes	21.8	0.1

Table 4. CT values obtained at optimal primer/probe concentration.

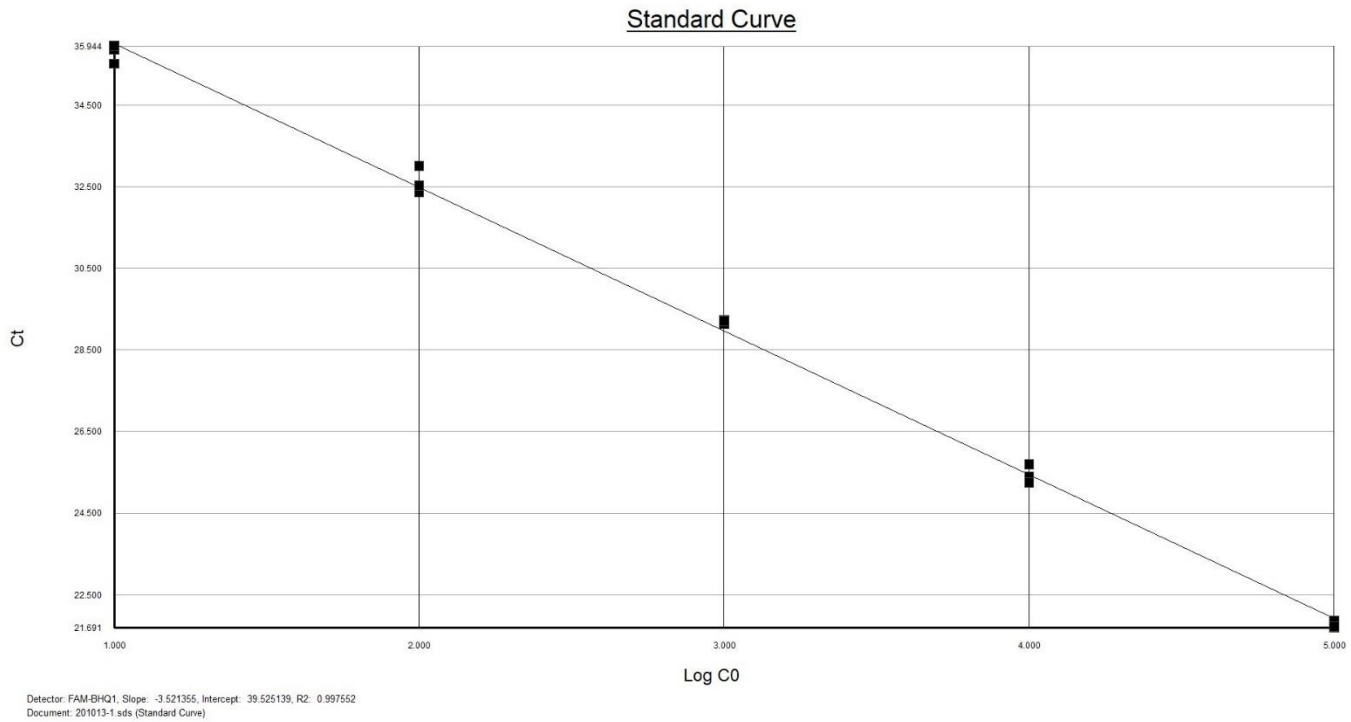

Figure 2. Standard curve of SYL000 based on 1, 10, 10^2, 10^3, 10^4 and 10^5 DNA target copies.

Slope	-3.5
Efficiency	92.4%
R^2	0.997

Table 5. Values obtained from the standard curve.

1.2.3. Influence of inhibiting factors present in environmental samples and repeatability

Performance of the test was tested on the influence of inhibiting factors present in three different kinds of environmental water samples (sandy, clayey or peaty bottoms) in three fold spiked with 1000 target DNA copies. Standard deviations, difference to spike and the standard error of mean were calculated to determine robustness, repeatability and measurement uncertainty.

Results:

No fluorescent output signal was detected in the environmental samples without added target organism DNA (spike). Spiked samples and the spike only analyses gave in all replicates a positive signal (**Table 6**). The difference between environmental samples and the spike only sample was less than 3%. Influence of chemical and biological factors present in environmental samples was not detected. The standard error of mean was 0.08 within these samples with an average of 24.7.

Sample	CT value	Standard deviation	ΔCT
Sandy	-	-	-
Clayey	-	-	-
Peaty	-	-	-
Sandy + spike	24.8	0.5	- 1.1%
Clayey + spike	24.7	0.2	- 0.7%
Peaty + spike	24.8	0.2	- 1.3 %
Spike-only	24.5	0.1	-

Table 6. CT values obtained with target organisms free environmental samples spiked with 1000 target copies.

1.2.4. Minimal filtration volume

The amount of eDNA that a target organism leaves in the environment depends on the type of target organism, environmental factors and season (CEN / TC230-water analysis, proposal documented in N 1229). To determine the minimal filtration volume, samples were taken at locations where : Ondatra zibethicus may occur, during the optimal seasons (spring, summer and autumn). Samples were taken with SYL001 - Environmental Sampling Kit and eDNA was isolated with SYL002 - Environmental DNA Isolation Kit.

Results:

Coming soon.

1.2.5. Sequence conformation of specificity

PCR products obtained were sequenced for conformation of identity of the formed product. If any product was formed with target organism free environmental samples, than these products were also be sequenced.

Results:

Target organism free environmental samples did not give any PCR product.

1.3. Summary of validation

1.3.1. Robustness

"Influence of temperature variations and inhibiting factors present in environmental samples on the performance and specificity of the primer set"

- Primers specific at temperature range: 50.0°C - 63.6°C (**Section 1.2.1**, **Table 3**).
- Differences (± 3%) due to influence of chemical and biological factors present in environmental samples on the performance of the test were not found. (**Section 1.2.3**, **Table 6**).
- Fluorescent output signals of positive samples is at least 100-fold stronger than the background.

1.3.2. Detection limit

"Limits (lower and upper limit) within which the analysis can be reliably applied"

- Limit of detection (LOD) for this kit was determined on 1 copy per reaction. Limit of quantification (LOQ) was also determined on 10 copies per reaction (**Section 1.2.2**, **Table 4**).

1.3.4. Efficiency

"The comparison of what is actually produced with what can be achieved with the same consumption of resources"

The efficiency of the primer set is 92.4 %, this means that the primer/probe mixture can be regarded as optimal (**Section 1.2.2**; **Table 5**).

1.3.5. Repeatability

"The degree of similarity between the results of measurements of the same measured quantity that were performed under different measurement conditions"

There was no difference (± 3%) between different kinds of environmental water samples (sandy, clayey or peaty bottoms) spiked with 1000 target DNA copies. (**Section 1.2.3**.; **Table 6**).

1.3.6. Correctness

"The ability of the method to do what it 'says' to do"

- The method did not give any other combined BLAST hit than the target organism *Ondatra zibethicus.*
- The method was able to detect all spiked target DNA in target organism free environmental samples (**Section 1.2.3.**; **Table 6**).
- The method did not give any signal in target organism free environmental samples (**Section 1.2.3.**; **Table 6**).
- The standard error of the mean based on the measured CT values from the spiked experiments (1000 target copies) was 0.08 on an average of 24.7.

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