Validation report:

Natrix natrix qPCR detection kit

with eDNA qPCR hot start mix

#SYL168

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1. Validation report *Natrix natrix* detection kit

1.1 In silico validation

Table 1: Forward primer *in silico* validation

Length	24
GC %	45
Stability	3.0
T_M (°C)	62
Target region	CytB (mtDNA)
Dimer	No
Run	No
Database hit	*Natrix natrix*

Table 2: Reverse primer *in silico* validation

Length (bp)	25
GC %	40
Stability	1.4
T_M (°C)	61
Target region	CytB (mtDNA)
Dimer	No
Run	No
Database hit	*Natrix natrix*

Table 3: Probe *in silico* validation

Length	28
GC %	53
T_M (°C)	73
Target region	CytB (mtDNA)
Dimer	No
Run	No
Fluorescence label	FAM

Table 4: Combined primers and probe *in silico* validation

PCR product size (bp)	110
Combined dimer formation	No
***In silico* PCR on Genbank**	*Natrix natrix*
Date of *In silico* PCR	December 2022

1.2 Experimental validation

All validation experiments were performed with the Sylphium qPCR mix and performed on Applied biosystems 7300 qPCR machines and Biometra gradient PCR machines.

1.2.1 Optimal annealing temperature primers

The annealing temperature has a strong influence on the performance, robustness and specificity of the primer set. To determine the optimal annealing temperature, a temperature gradient PCR between 50°C and 72°C was performed in eight steps.

Results:

The expected product was formed between 50.0°C and 67,6.0°C. No non-specific additional fragments were formed at the temperatures tested. No primer dimers were formed at any temperature tested. The optimum annealing temperature was determined to be 60.0°C (see Table 5).

Table. 5: Temperature gradient PCR on DNA of *Natrix natrix.*

Annealing temp.	**50.0°C**	**50.7°C**	**52.1°C**	**54.4°C**	**57.0°C**	**59.7°C**	**62.3°C**	**65.0°C**	**67.6°C**	**69.9°C**	**71.3°C**	**72.0°C**
Expected fragment	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**	**No**	**No**	**No**
Primer dimer	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**
Additional fragments	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**	**No**

1.2.2 detection limit, fluorescence output signal and efficiency

Standard solutions with 10^7, 10^5, 10^4, 10^3, 10^2, 10, 1 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:

The detection limits (low and high) for qualitative detection was determined between 1-10 and >10^7 target copies per reaction. The detection limits (low and high) for quantitative detection was determined between 1-10 and >10^7 target copies per reaction. The fluorescence output signal was at least 100 (8 for positive sample, 0,01 for a negative sample) times stronger than the background signal. (fig. 1). The efficiency of the test was above 90%. Which means that the efficiency of the primer/probe mixture can be regarded optimal (Fig. 2, table 7).

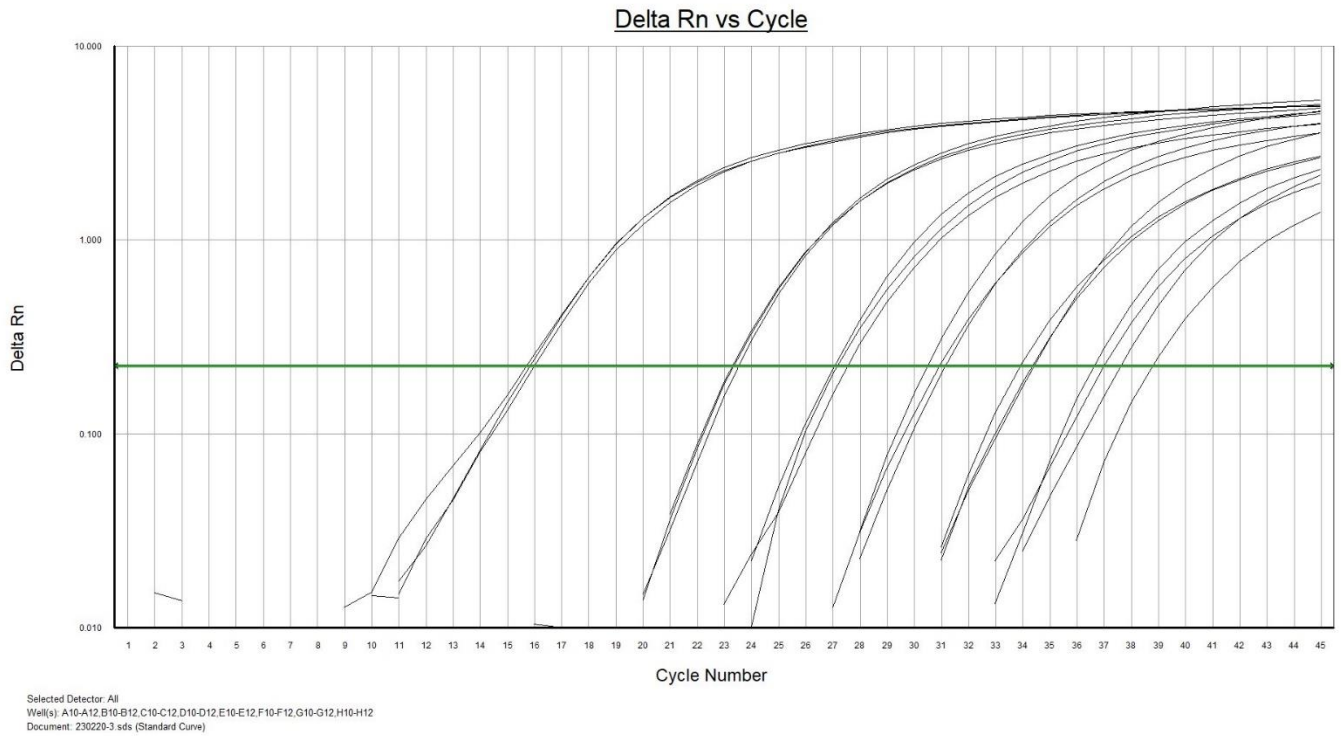

Figure. 1: Obtained fluorescent signal at optimal primer/probe concentration at a annealing temperature of 60°C.

Table 6: CT values obtained at optimal primer/probe concentration.

target DNA copy	Target detected	CT value	Standard deviation
0	Yes	n.a.	n.a.
1	1 of 3	n.a.	n.a.
10	Yes	37.1	0.5
10^2	Yes	34.2	0.3
10^3	Yes	30.8	0.4
10^4	Yes	27.2	0.2
10^5	Yes	23.3	0.1
10^7	Yes	15.8	0.2

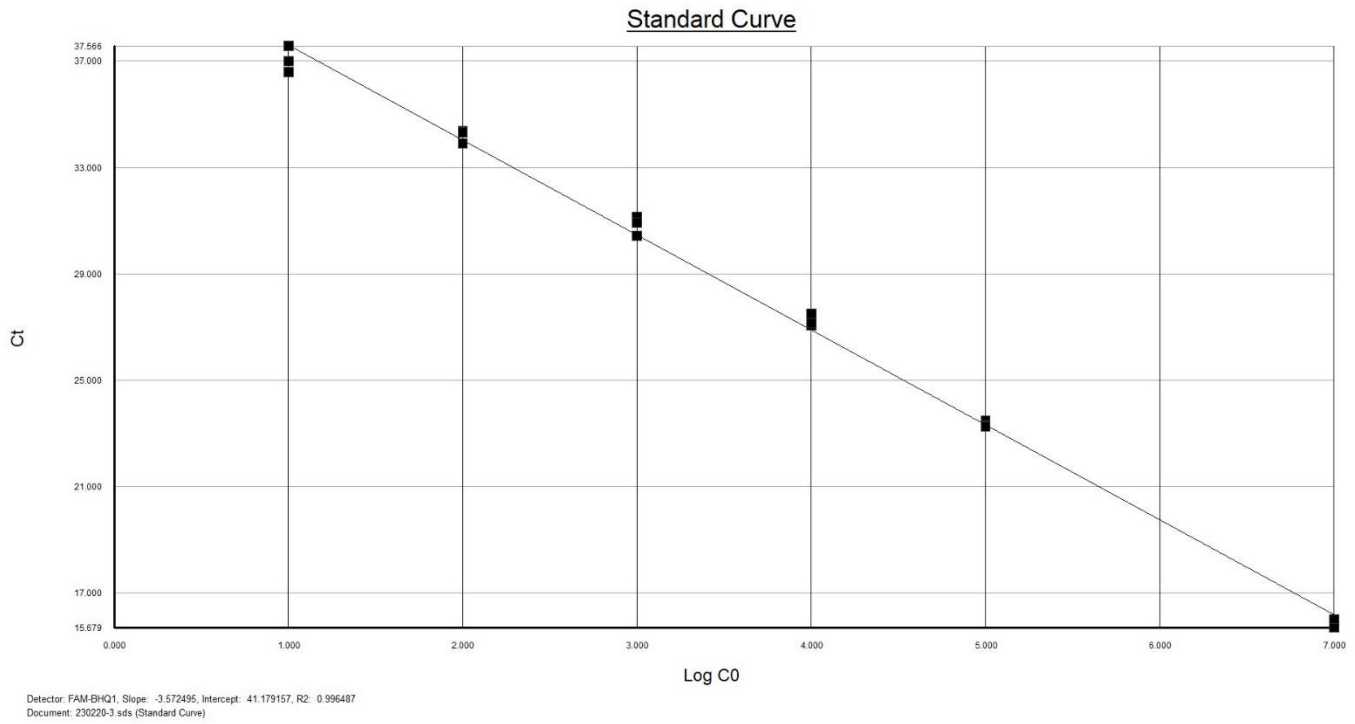

Fig. 2: Standard curve of SYL168 based on 10, 10^2, 10^3, 10^4, 10^5 and 10^7 DNA target copies

Table 7: Values obtained from the standard curve

Slope	-3.57
Efficiency	90.7%
R^2	0.996

1.2.3 Influence of inhibiting factors present in environmental samples and repeatability

Performance of the assay 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. The ΔCT between spiked samples and spike only was determined and should be less than 2. Standard deviations 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. (see table 8). In all cases the ΔCT was less than 2.

Table 8: CT values obtained with target organisms free environmental samples spiked with 1000 target copies.

Sample	CT value	Standard deviation	ΔCT
Sandy	-	-	-
Clayey	-	-	-
Peaty	-	-	-
Sandy + spike	29.8	0.0	0.1
Clayey + spike	29.8	0.2	0.1
Peaty + spike	29.7	0.0	0.0
Spike only	29.7	0.1	-

1.2.4 Detection conformation of *Natrix natrix* in environmental samples

No field validation tests have been conducted yet. These tests are planned for the coming season. We do not foresee any problems with the detection of *Natrix natrix*. Based on the field experiments with *Natrix maura*, it can be stated that snakes in an aquatic environment have good detectability.

1.2.5 Sequence conformation of specificity.

No field validation tests have been conducted yet. 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°C – 67,6°C (section 1.2.1, table 5)
- Influence of chemical and biological factors present in environmental samples on the performance of the test were not found. (section 1.2.3, table 8)
- Fluorescent output signals of positive samples is at least 100 fold stronger than the background

1.3.2 Detection limit

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 95.7 %, this means that the primer/probe mixture can be regarded as optimal. (section 1.2.2, table 7)

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 (ΔCT <2) between different kinds of environmental water samples (sandy, clayey or peaty bottoms) spiked with 1000 target DNA copies. (section 1.2.3, table 8)

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 *Natrix natrix*
- The method was able to detect all spiked target DNA in target organism free environmental samples (section 1.2.3, table 8)
- The method did not give any signal in target organism free environmental samples (section 1.2.3, table 8)
- The standard error of the mean based on the measured CT values from the spiked experiments (1000 target copies) was 0.09 on an average of 27.8.