Validation report:

Natrix maura qPCR detection kit

with eDNA qPCR hot start mix

#SYL127

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1. Validation report *Natrix maura* detection kit

1.1 In silico validation

Table 4: Forward primer *in silico* validation

Length	20
GC %	50
Stability	2.6
TM (°C)	60
Target region	CytB (mtDNA)
Dimer	No
Run	No
Database hit	*Natrix maura*

Table 5: Reverse primer *in silico* validation

Length (bp)	20
GC %	45
Stability	2.1
TM (°C)	60
Target region	CytB (mtDNA)
Dimer	No
Run	No
Database hit	*Natrix sp.*

Table 6: Probe *in silico* validation

Length	27
GC %	48
T_M (°C)	69
Target region	CytB (mtDNA)
Dimer	No
Run	No
Fluorescence label	FAM

Table 7: Combined primers and probe *in silico* validation

PCR product size (bp)	118
Combined dimer formation	No
Combined 1000 BLAST® analyses of both primers	*Natrix maura*
Date of BLAST® analyses	*November 2018*

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 Gradient PCR

Influence of the annealing temperature on the performance and specificity of the primer set was tested with a temperature gradient between 47.5°C to 72.5°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 47.5°C and 68.1°C. Nonspecific additional fragments were formed at 47.5°C and 49.7°C. No primer dimer were formed at any tested temperature. The optimal annealing temperature was between 55.5°C and 64.4°C (table 8).

Table. 8: Temperature gradient PCR on genomic DNA of *Natrix maura*.

Annealing temp.	**47.5°C**	**49.8°C**	**55.5°C**	**58.5°C**	**61.5°C**	**64.4°C**	**70.1°C**	**72.5°C**
Expected fragment	Strong	Strong	Strong	Strong	Strong	Strong	No	No
Primer dimer	No	No	No	No	No	No	No	No
Additional fragments	Yes	Yes	No	No	No	No	No	No

1.2.2 detection limits, fluorescence output signal and efficiency

Standard solutions with 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:

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. (Fig. 2, table 9).

The fluorescence output signal was at least 100 (5 for positive sample, 0,05 for a negative sample) times stronger than the background signal. (fig. 1, table 8)

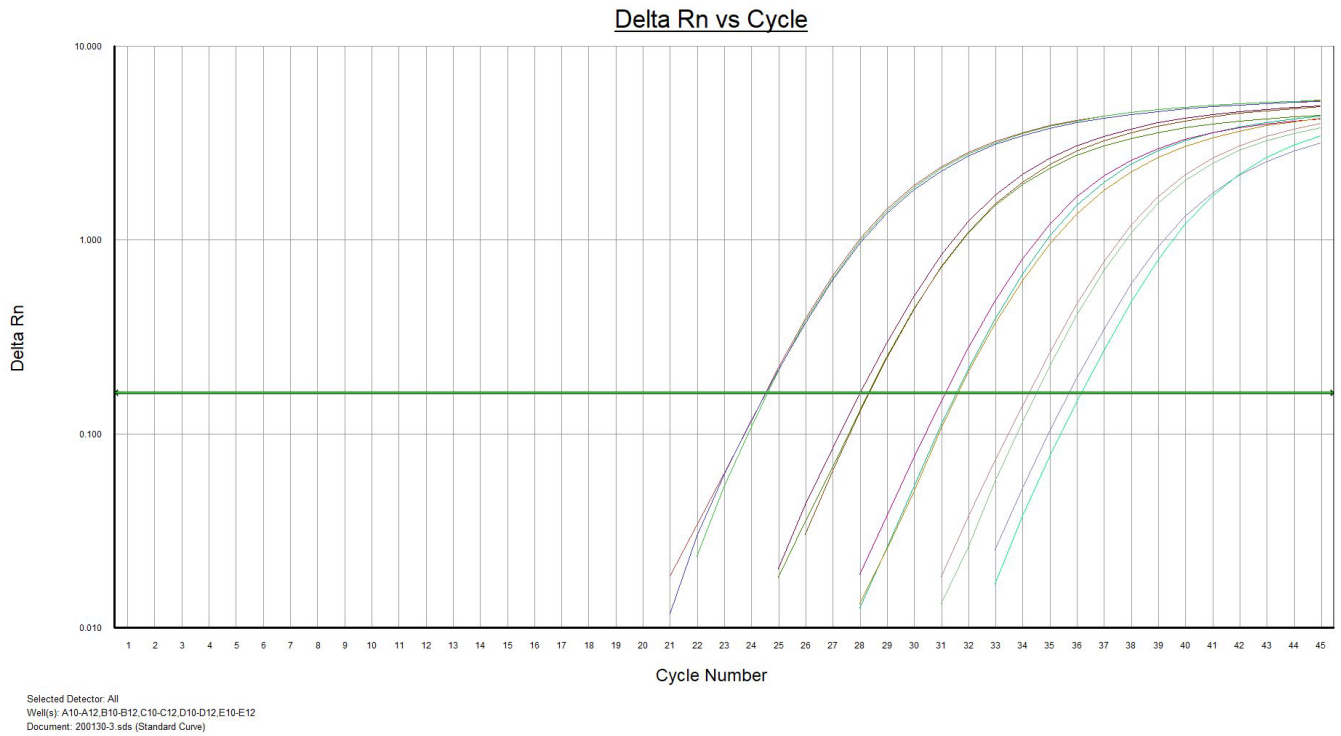

Figure. 1: Obtained fluorescent signal at optimal primer/probe concentration at a annealing temperature of 60°C.

Table 8: CT values obtained at optimal primer/probe concentration.

target DNA[1] copy	Target detected	CT value	Standard deviation
0	No	-	-
1	Yes	n.a.	-
10	Yes	33.7	0.2
10^2	Yes	31.8	0.3
10^3	Yes	29.2	0.1
10^4	Yes	25.9	0.1

[1] Estimated by gel electrophoresis

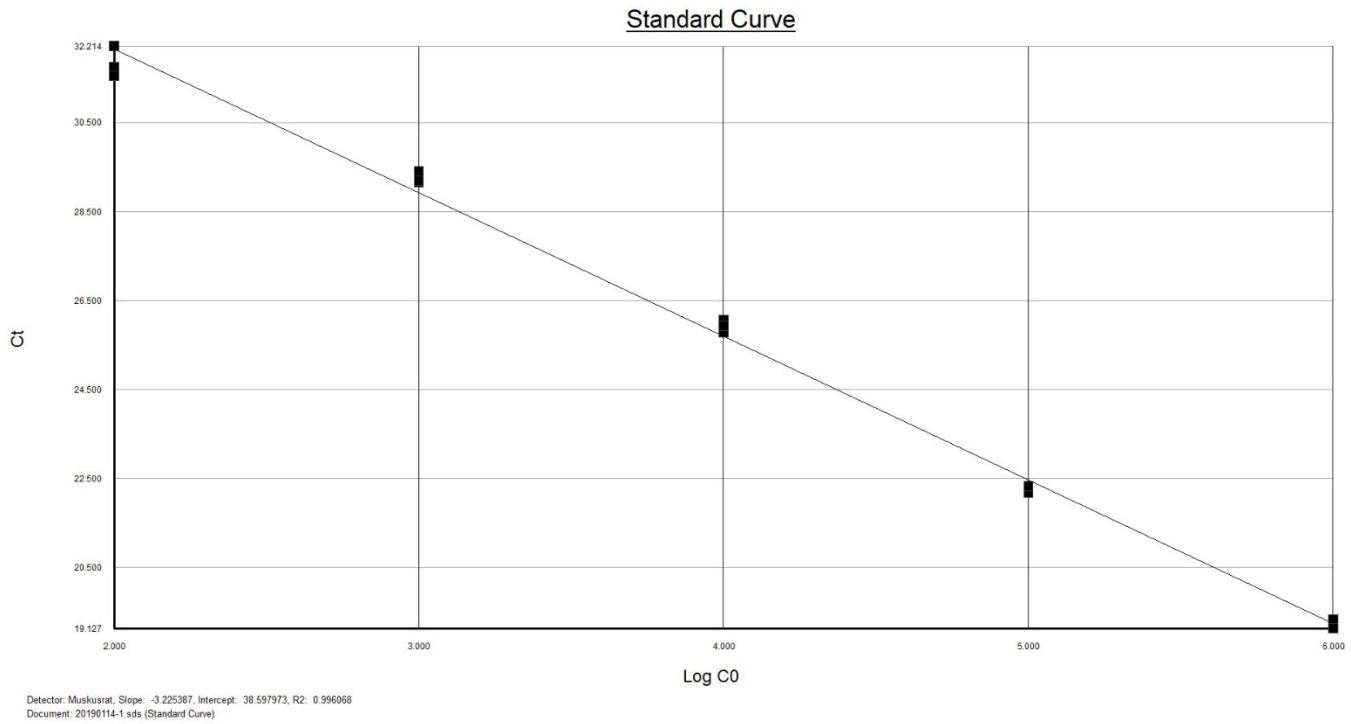

Fig. 2: Standard curve of SYL127 based on 10, 10^2, 10^3 and 10^4 DNA target copies

Table 9: Values obtained from the standard curve

Slope	-3.23
Efficiency	104.0%
R^2	0.996

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, one way ANOVA 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 10). There were no statistically significant differences between group means as determined by one-way ANOVA ($F(3,8) = 2.500$, $p = .13$) Influence of chemical and biological factors present in environmental samples was not detected. The standard error of mean was 0.21 within these samples with an average of 30.0

Table 11: CT values obtained with target organisms free environmental samples spiked with 1000 target copies.

Sample	CT value	Standard deviation
Sandy	-	-
Clayey	-	-
Peaty	-	-
Sandy + spike	30.7	1.2
Clayey + spike	30.0	0.3
Peaty + spike	29.9	0.2
Spike only	29.3	0.1

1.2.4 Detection conformation in environmental samples

Conformation of detection with the test was performed on samples form locations where the presence of *Natrix maura* was suspected. Samples were taken using SYL001 - Environmental sampling kit and eDNA was isolated using SYL002 -Environmental DNA isolation kit.

Results:
The kit SYL127 – *Natrix maura* detection kit was able to detect *Natrix maura* in environmental samples from the Jura region in France. On average, a positive sample gave 1 molecule *Natrix maura* DNA in 11 ml water. See appendix A.

4.2.5 Sequence conformation of specificity.

PCR products obtained from environmental samples 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. PCR products obtained from environmental samples were sequenced in triplo via the Sanger sequencing method. The obtained sequences were identical to each other and Blast ® hits confirmed identity of the target organism.

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: 47.5°C – 64.4°C (section 1.2.1, section 1.2.2, table 8, table 9)
- Statistical differences (p>0.05) due to influence of chemical and biological factors present in environmental samples on the performance of the test were not found. (section 1.2.2, table 11)
- Fluorescent output signals of positive samples is at least 100 fold stronger than the background

1.3.2 Detection limits

"Lowest limits in which the analysis can be reliably applied"

Limit of detection (LDqPCR) for this kit was determined on 1 copies per reaction. Limit of quantification (LQqPCR) was also determined on 10 copies per reaction. (section 1.2.2, table 8)

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 104%, this means that the primer/probe mixture can be regarded as optimal. (section 1.2.2, table 9)

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 statistical differences (p>0.05) between different kinds of environmental water samples (sandy, clayey or peaty bottoms) spiked with 1000 target DNA copies. (section 1.2.3, table 11)

1.3.6 Correctness

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

- The test was able to detect *Natrix maura* DNA in environmental samples from locations were the presence of *Natrix maura* was suspected. (section 1.2.6)
- The method did not give any other combined BLAST hit than the target organism *Natrix maura*
- The method was able to detect all spiked target DNA in target organism free environmental samples (section 1.2.3, table 10)
- The method did not give any signal in target organism free environmental samples (section 1.2.3, table 11)
- The standard error of the mean based on the measured CT values from the spiked experiments (1000 target copies) was 0.4 on an average of 30.6.

Appendix A:

Analysis report

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Client	Own research
Contact person	n.a.
Number of samples	4 sterivex filters
Organism(s) to be detected	*Natrix maura*
Report Date	10-6-2020
Performed by	Jan Warmink

Monster code	Location	Volume	description
NmRS	Sorne, Macornay, France	280 ml	Snakes observed during sampling
NmBG	Bief Grougneau, Macornay, France	280 ml	No snakes observed
E2477	Hunze, Gieterveen, Netherlands	250 ml	Species does not occur here
E2478	Hunze, Gieterveen, Netherlands	250 ml	Species does not occur here

Table 1: Samples Supplied.

Implementation and quality assurance:
The analyses of the samples were carried out in eightfold. A sample is tested positive if at least one of these analyses gives a positive signal. As controls were used:

- Efficiency and inhibition control (RIC): Xenobiotic DNA has been added to the samples as a control. This check excludes false negative PCR results, which are caused by disturbing factors in the DNA isolate. This check also determines the isolation efficiency of the procedure carried out. When disturbing factors are found, the experiment is repeated at sample dilutions of 2x, 4x and 8x. Based on these results, it is decided on which dilution the *Natrix maura* analysis is carried out.
- Procedure blank: Only preservative solution used, that goes through all the isolation and analysis steps. This check shows possible contamination with DNA during processing.
- PCR positive control: *Natrix maura* DNA added to PCR mix. This check excludes false negative PCR results, due to errors in the PCR process.
- PCR negative control: No sample or DNA added. This is an additional check for false positive results by contamination.

Results:
The sample NmRS was tested positive for the presence of *Natrix maura* DNA (Table 2). The other samples were tested negative. The positive checks appear to be positive in all cases. The negative controls gave negative results in all cases.

Monster code	Results *Natrix maura*	Procedure blank	Inhibition Control	PCR negative control	PCR positive control
NmRS	3/8	Ok	Ok	Ok	Ok
NmBG	0/8	Ok	Ok	Ok	Ok
E2477	0/8	Ok	Ok	Ok	Ok
E2478	0/8	Ok	Ok	Ok	Ok

2: PCR results analysis samples.

Conclusion:
The sample NmRS was tested positive for the presence of *Natrix maura* DNA (Table 2). The other samples were tested negative. These checks indicate that there were no disturbing factors or DNA contaminations from the target species. False negative and false positive results can be excluded.